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

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

### *2.2 Ripeness Index*

The ripening index (RI) was determined according to the method described by the International Olive Oil Council (Salvador, Aranda, & Fregapane, 2001), and the ripening developmental period was split into four stages according to the work of Conde, Delrot & Gerós (2008). For this purpose, 100 drupes were randomly sampled as previously described and divided into color groups according to the spread of pigmentation on the pericarp and mesocarp of the fruit.

The scale for color grouping varied from 0 (intense green) to 7 (100% colored of pericarp and mesocarp). The index was calculated as the weighted average number of drupes within each subset of samples.

*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 a 10 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. New models relative to total chlorophylls, total carotenoids, total anthocyanins, rutin, and firmness were built by combining data from all three cultivars and the total sample set of data (n = 33) both for the calibration and validation procedures which, because of the small number of data, were carried out only using leave-one-out cross-validation method (Dardenne, 2010). Each number of sample was represented by 10 grouped drupes; two NIR spectra each drupe equal to 660 total spectra in the dataset. The same dataset of spectra (n = 33) was used to perform the validation of specific models previously assessed (Bellincontro et al, 2012) for oleuropein, verbascoside 3,4-DHPEA-EDA, and total phenols. 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 (Supplementary Fig. 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 (Supplementary Fig. 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 (Supplementary Fig. 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 (Fig. 1). 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). 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. Principal component analysis (PCA) calculated for all spectral datasets discriminated the three cultivars, and significant separation was obtained for Buscionetto (Supplementary Fig. 2). 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 quantitative information in the detected results (Table 3, 4). The values for calibration and cross-validation of the physical and biochemical parameters in the olive drupes during the ripening period are presented in Table 3, whereas the values of biochemical parameters that were used to validate the previous calculations already performed for the other cultivars are presented in Table 4 (Bellincontro et al., 2012). The highest correlation ($R^2 = 0.997$) value was obtained for firmness (Fig. 2a), whereas slightly lower values were obtained for total phenol and verbascoside content ($R^2 = 0.965$ for both; Fig. 2b and Fig. 2d, respectively), 3,4-DHPEA-EDA ($R^2 = 0.934$; Fig. 2c), rutin ($R^2 = 0.925$; Fig. 2f) and total anthocyanins ($R^2 = 0.910$; Fig. 2h). Lower but still acceptable $R^2$ were obtained for the calibration models for oleuropein ($R^2 = 0.897$; Fig. 2e),

total carotenoids ($R^2$ = 0.887; Fig. 2i) and total chlorophylls ($R^2$ = 0.868; Fig. 2g). The RMSEC index, expressed as milligrams per gram of fresh weight, varied from the lowest value of 0.002 for total carotenoids to the highest of 1.44 for 3,4-DHPEA-EDA, whereas the number of LVs was in the range of 4-8, except for verbascoside, where it was of 10. The leave-one-out cross-validation method was used to evaluate the predictive ability of the PLS models. This method is considered appropriate for a limited sample data set (Dardenne, 2010).

The cross-validation of PLS models was characterized by a reduction of the $R^2cv$ coefficient, particularly for total anthocyanins and rutin ($R^2cv$ = 0.80 and $R^2cv$ = 0.83, respectively). However, the RMSEC and RMSECV indices for total chlorophyll and carotenoids had very similar values, indicating that an optimum number of factors were included in the models. Interestingly, the cross-validated model for the firmness parameter still had a high $R^2cv$ value (0.99) and low error. Oleuropein, verbascoside, 3,4-DHPEA-EDA and total phenols were validated using PLS models already created by Bellincontro et al.[36] in the cvs Moraiolo, Dolce d'Andria and Nocellara Etnea. The validation showed a 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 (García, Seller, & Pérez-Camino, 1996). Kavdir et al. (2009) and, more recently Beghi et al. (2013), have correlated olive firmness measured with a portable penetrometer to reflectance spectra; in the first case the spectra were detected by a FT-NIR spectrometer (ranging from 800 to 2500 nm), whereas a vis/NIR spectrophotometer (ranging from 400 to 1000 nm) was used in the second. In Kavdir's work[40] the $R^2$ results obtained in calibration and in cross-validation were of 0.75 and 0.68, respectively, whereas they were equal to 0.68 and 0.66, respectively, in Beghi's work (2013).

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

before the oil extraction process, and obtained calibration and validation $R^2$ values ranging from 0.86 to 0.88 for spectroscopic techniques.

In terms of spectra contribution to the PLS modeling, our previous works already have established significant correlations about prediction of total and specific phenols (Bellincontro et al., 2012). Here the interest is addressed to evaluate NIR spectra performance in relation to the new models carried out for chlorophylls, carotenoids, and firmness. In supplementary figure S3, regression coefficients for chlorophylls and carotenoids models (a), and for firmness model (b) have been reported. Even though it is largely known the stronger absorption response of chlorophylls and carotenoids in the visible range of light spectra, literature reports significant results in correlation obtained by applying NIR spectroscopy to predict the content of these pigments, mostly carotenoids (McGoverin et al., 2010). Both for chlorophylls and carotenoids (Figure S3a), correlation peaks were found at 1198 nm, 1725 nm, 1930 nm, 2255 nm, and 2280 nm. By working on kale for prediction of specific carotenoids content, Chen et al. (2009) found significant correlations at 1235 nm, 1770 nm, 2176 nm, and 2376 nm. The wavelength range between 1100-1300 nm is related to the second overtone and 1700-1900 nm to the first overtone of C-H bonds, while 2000-2500 nm to the deformation of C-H bonds. The quite similar behavior that we observed both for chlorophylls and carotenoids is probably due to the strong correlation between them (Sims & Gamon, 2000). Regarding firmness (Figure S3b), PLS regression peaks were individuated at 1248 nm, 1449 nm, 1758 nm, 1917 nm, 1990 nm, and 2238 nm. Negative correlation related to absorption at 1449 and 1917 nm are for sure dominated by the H2O response, respectively associated to the first overtone of the symmetric and asymmetric OH stretching and/or combination bands (1450 nm), and to the combination of the OH stretching band and to the OH bending band (1920 nm). Absorptions at 1248 nm and 1758 are referred to the second and to the first overtone of the CH stretching vibrations of CH3, CH2, and CH=CH, respectively. It is known that fruit firmness is affected by dry matter (McGlone & Kawano, 1998), and in olives dry matter is in turn related to the oil content

(Cayuela & Pérez-Camino, 2010). On this basis, we may consider that firmness detection can be an indirect index which helps to establish yield prediction and oil quality.

The obtained results confirmed the ability of NIRS-AOTF to predict total phenol content and specific metabolites, as previously reported in other cultivars. The use of fruits collected from the plants of cultivars defective in specific phenotypical characters and, therefore, considered as natural mutants allowed for the robustness of the procedure to be assessed. The cultivars diverged in phenol composition: Leccino fruit contains a high 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., 2011). 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). The opportunity to monitor olive firmness through the in-field use of a rapid and accurate non-destructive tool, is strictly related to the fruit

maturity identification. More in detail, the relationship among textural properties, water content and water status, and cell wall degradation metabolisms (Brummel, 2006) suggests that fruit firmness can be considered a significant indicator of the cellular extractability. This has a strong involvement in the olive oil richness and final quality. 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 two cultivars, 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 considering a large biological variability. 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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## Appendix A. Supplementary data

This document contains Supplementary Fig. S1 and S2.

## References

Alagna, F., Mariotti, R., Panara, F., Caporali, S., Urbani, S., Veneziani, G., et al. (2012). Olive phenolic compounds: metabolic and transcriptional profiling during fruit development. *BMC Plant Biology*, *12*, 162–183.

Aparicio-Ruiz, R., Gandul-Rojas, B., & Roca, M. (2009). Pigment profile in non-Spanish olive varieties (*Olea europaea* L. var Coratina, Frantoio, and Koroneiki. *Journal of Agricultural and Food Chemistry, 57*, 831–836.

Bartolini, G. OLEA databases, URL (http://www.oleadb.it/), most recent access data on 3rd of June 2015.

Beghi, R., Giovenzana, V., Civelli, R., Cini, E., & Guidetti, R. (2013). Characterisation of olive fruit for the milling process by using visible/near infrared spectroscopy. *Journal of Agricultural Engineering*, *XLIV:e8*, 56–61.

Bellincontro, A., Taticchi, A., Servili, M., Esposto, S., Farinelli, D., & Mencarelli, F. (2012). Feasible application of a portable NIR-AOTF tool for on-field prediction of phenolic compounds

during the ripening of olives for oil production. *Journal of Agricultural and Food Chemistry, 60,* 2665–2673.

Bonoli, M., Bendini, A., Cerretani, L., Lercker, G., & Gallina-Toschi, T. (2004). Qualitative and semiquantitative analysis of phenolic compounds in extra virgin olive oils as a function of the ripening degree of olive fruits by different analytical techniques. *Journal of Agricultural and Food Chemistry, 52,* 7026–7032.

Brummell, D. A. (2006). Cell wall disassembly in ripening fruit. *Functional Plant Biology, 33,* 103–119.

Camposeo, S., Vivaldi, G. A., & Gattullo, C. E. (2013). Ripening indices and harvesting times of different olive cultivars for continuous harvest. *Scientia Horticulturae, 151,* 1–10.

Carrasco-Pancorbo, A., Gómez-Caravaca, A. M., Cerretani, L., Bendini, A., Segura-Carretero, A., & Fernández-Gutiérrez, A. (2006). Rapid quantification of the phenolic fraction of Spanish virgin olive oils by capillary electrophoresis with UV detection. *Journal of Agricultural and Food Chemistry, 54,* 7984–7991.

Cayuela, J. A., & Pérez-Camino, M. C. (2010). Prediction of quality of intact olives by near infrared spectroscopy. *European Journal of Lipid Science and Technology, 112,* 1209−1217.

Chen, X., Wub, J., Zhoua, S., Yanga, Y., Nib, X., Yangb, J., Zhuc, Z., & Chunhai, S., (2009). Application of near-infrared reflectance spectroscopy to evaluate the lutein and β-carotene in Chinese kale. *Journal of Food Composition and Analysis, 22(2),* 148–153.

Chen, L., & Opara, U. L. (2013). Texture measurement approaches in fresh and processed foods - A review. *Food Research International, 51(2),* 823–835.

Conde, C., Delrot S., & Gerós H. (2008). Physiological, biochemical and molecular changes occurring during olive development and ripening. *Journal of Plant Physiology, 165,* 1545–1562.

Dardenne, P. (2010). Some considerations about NIR spectroscopy: closing speech at NIR-2009. NIR News, *21,* 8–14.

Galtier, O., Dupuy, N., Le Dréau, Y., Olivier, D., Pinatel, C., Kister, J., et al. (2007). Geographical origins compositions of virgin olive oils determined by chemometric analysis of NIR spectra. *Analytica Chimica Acta*, *595*, 136–144.

García, J.M., & Yousfi, K. (2006). The postharvest of the mill olives. *Grasas Y Aceites*, *57(1)*, 14–24.

García, J. M., Seller, S., & Pérez-Camino, M. C. (1996). Influence of fruit ripening on olive oil quality. *Journal of Agricultural and Food Chemistry 44*, 3516–3520.

Giovenzana, V., Beghi, R., Civelli, R., Marai, S., & Guidetti, R. (2015). Postharvest Characterization of Olive Oil Fruits Texture by NIR and Vis/NIR Spectroscopy. *Chemical Engineering Transactions*, *44*, 61–66.

Inglese, P., Famiani, F., Galvano, F., Servili, M., Esposto, S., & Urbani, S. (2011). Factors affecting extra virgin olive oil composition. *Horticultural Reviews*, *38*, 83–147.

Kavdir, I., Buyukcan, M.B., Lu, R., Kocabiyik, H., & Seker, M. (2009). Prediction of olive quality using FT-NIR spectroscopy in reflectance and transmittance modes. *Biosystems Engineering*, *103*, 304–312.

Jiménez, A., Rodríguez, R., Fernández-Caro, I., Guillén, R., Fernández-Bolaños, J., & Heredia, A. (2001a). Olive fruit cell wall: Degradation of cellulosic and hemicellulosic polysaccharides during ripening. *Journal of Agricultural and Food Chemistry*, *49*, 2008–2013.

Jiménez, A., Rodríguez, R., Fernández-Caro, I., Guillén, R., Fernández-Bolaños, J., & Heredia, A. (2001b). Olive fruit cell wall: Degradation of pectic polysaccharides during ripening. *Journal of Agricultural and Food Chemistry*, *49*, 409–415.

Lichtenthaler, H. K., & Wellburn, A. R. (1983). Determination of total carotenoids and chlorophyll a and b of leaf extract in different solvents. *Biochemical Society Transactions*, *11*, 591–592.

Martinelli, F., & Tonutti, P. (2012). Flavonoid metabolism and gene expression in developing olive (*Olea europaea* L.) fruit. *Plant Biosystems*, *146(1)*, 1–7.

Marquez, A. J., Diáz, A. M., & Reguera, M. I. P. (2005). Using optical NIR sensor for on-line virgin olive oils characterization. *Sensors Actuators B: Chemical, 107*, 64−68.

Mínguez-Mosquera, M. I., & Gallardo-Guerrero, M. L. (1991). Disappearance of chlorophylls and carotenoids during the ripening of the olive. *Journal of the Science of Food and Agriculture, 69,* 1–6.

McGlone, V. A., & Kawano, S. (1998). Firmness, dry-matter and soluble-solids assessment of postharvest kiwifruit by NIR spectroscopy. *Postharvest Biology and Technology, 13,* 131–141.

McGoverin, C. M., Weeranantanaphan, J., Downey, G., & Manley, M. (2010). Review: the application of near infrared spectroscopy to the measurement of bioactive compounds in food commodities. *Journal of Near Infrared Spectroscopy,* 18, 87–111.

Montedoro, G., Servili, M., Baldioli, M., Selvaggini, R., Miniati, E., & Macchioni, A. (1993). Simple and hydrolyzable compounds in virgin olive oil. 3. Spectroscopic characterization of the secoiridoids derivatives. *Journal of Agricultural and Food Chemistry, 41*, 2228−2234.

Moran, R. (1982). Formulae for determination of chlorophyllous pigments extracted with N,N-Dimethylformamide. *Plant Physiology, 69,* 1376–1381.

Moyano, M. J., Melendez-Martinez, A. J., Alba, J., & Heredia, F. J. (2008). A comprehensive study on the colour of virgin olive oils and its relationship with their chlorophylls and carotenoids indexes (I): CIEXYZ non-uniform colour space. *Food Research International, 41,* 505–512.

Nicolai, B., Beullens, K., Bobelyn, E., Peirs, A., Saeys, W., Theron, K. I., et al. (2007). Non-destructive measurements of fruit and vegetable quality by means of NIR spectroscopy: A review. *Postharvest Biology and Technology, 46*, 99−118.

Pasqualone, A., Di Rienzo, V., Blanco, A., Summo, C., Caponio, F., & Montemurro, C. (2012). Characterization of virgin olive oil from Leucocarpa cultivar by chemical and DNA analysis. *Food Research International, 47(2),* 188–193.

585 Rolle, L., Torchio, F., Zeppa, G., & Gerbi, V. (2009). Relations between break skin force and
586 anthocyanin extractability at different stages of ripening. *American Journal of Enology and*
587 *Viticulture, 60*, 93–97.
588 Romani, A., Mulinacci, N., Pinelli, P., Vinciert, F., & Cimato, A. (1999). Polyphenolic content in
589 five Tuscany cultivar of *Olea europaea* L. *Journal of Agricultural and Food Chemistry*, *47*,
590 964−967.
591 Rotondi, A., Bendini, A., Cerratani, L, Mari, M., Lercker, G., & Gallina Toschi, T. (2004). Effect of
592 olive ripening degree on the oxidative stability and organoleptic properties of cv. Nostrana di
593 Brisighella extra virgin olive oil. *Journal of Agricultural and Food Chemistry*, *52,* 3649-3654.
594 Salvador, M., Aranda, F., Fregapane, G. (2001). Influence of fruit ripening on "Cornicabra" virgin
595 olive oil quality. A study of four successive crop seasons. *Food Chemistry*, *73*, 45–53.
596 Santos, O. A., & Kaye, O. (2005). Grapevine water potential based upon near infrared spectroscopy.
597 *Scientia Agricola*, *66*, 287−292.
598 Selvaggini, R., Servili, M., Urbani, S., Esposto, S., Taticchi, A., & Montedoro, G. (2006).
599 Evaluation of phenolic compounds in virgin olive oil by direct injection in high-performance liquid
600 chromatography with fluorometric detection. *Journal of Agricultural and Food Chemistry*, *54*,
601 2832−2838.
602 Servili, M., & Montedoro, G. (2002). Contribution of Phenolic Compounds to Virgin Olive Oil
603 Quality European. *European Journal of Lipid Science and Technology, 104(9–10)*, 602–613.
604 Servili, M., Esposto, S., Fabiani, R., Urbani, S., Taticchi, A., Mariucci, F., et al. (2009). Phenolic
605 compounds in olive oil: antioxidant, health and sensory activities according to their chemical
606 structure. *Inflammopharmacology*, *17*, 1−9.
607 Sims, D. A., & Gamon, J. A. (2002). Relationships between leaf pigment content and spectral
608 reflectance across a wide range of species, leaf structures and developmental stages. *Remote*
609 *Sensing of Environment, 81*, 337 – 354

Tuck, K. L., & Hayball, P. J. (2002). Major phenolic compounds in olive oil: metabolism and health effects. *Journal of Nutrional Biochemistry, 13*, 636–644.

Vierhuis, E., Servili, M., Baldioli, M., Schols, H. A., Voragen, A. G. J., & Montedoro, G. (2001). Effect of enzyme treatment during mechanical extraction of olive oil on phenolic compounds and polysaccharides. *Journal of Agricultural and Food Chemistry, 49*, 1218–1223.

Wesley, I. J., Barnes, R. J., & McGill, A. E. J. (1995). Measurement of adulteration of olive oils by near-infrared spectroscopy. *Journal of the American Oil Chemists' Society, 72*, 289–292.

## Figure captions

Figure 1. 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.

Figure 2. Scatter plots compared to the predictive models for the drupe firmness (**a**), total phenols (**b**), DHPEA-EDA (**c**), verbascoside (**d**), oleuropein (**e**), rutin (quercetin-3-O-rutinoside) (**f**), total chlorophylls (**g**), total anthocyanins (**h**) and total carotenoids (**i**) for the global data set of olive samples (sum of the three cultivars). For each compound measured, experimental values are plotted versus predicted values.