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Feasible Application of a Portable NIR-AOTF Tool for On-Field Prediction of Phenolic Compounds during the Ripening of Olives for Oil Production

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ABSTRACT: Olive fruits of three different cultivars (Moraiolo, Dolce di Andria, and Nocellara Etnea) were monitored during ripening up to harvest, and specific and total phenols were measured by HPLC (High Pressure Liquid Chromatography). On the same olive samples (n = 450), spectral detections were performed using a portable NIR (Near Infrared)-AOTF (Acousto Optically Tunable Filter) device in diffuse reflectance mode (1100−2300 nm). Prediction models were developed for the main phenolic compounds (e.g., oleuropein, verbascoside, and 3,4-DHPEA-EDA) and total phenols using Partial Least Squares (PLS). Internal cross-validation (leave-one-out method) was applied for calibration and prediction models developed on the data sets relative to each single cultivar. Validation of the models obtained as the sum of the three sample sets (total phenols, n = 162; verbascoside, n = 162; oleuropein, n = 148; 3,4-DHPEA-EDA, n = 162) were performed by external sets of data. Obtained results in term of R^2 (in calibration, prediction and cross-validation) ranged between 0.930 and 0.998, 0.874−0.942, and 0.837−0.992, respectively. Standard errors in calibration (RMSEC), cross-validation (RMSECV), and prediction (RMSEP) were calculated obtaining minimum error in prediction of 0.68 and maximum of 6.33 mg/g. RPD ratios (SD/SECV) were also calculated as references of the model effectiveness. This work shows how NIR-AOTF can be considered a feasible tool for the on-field and nondestructive measurement of specific and total phenols in olives for oil production.

KEYWORDS: *olive fruits, total phenols, oleuropein, verbascoside, seociridoidids, NIR-AOTF spectroscopy, partial least square regression (PLSR)*

INTRODUCTION

The determination of the optimal fruit ripening stage in virgin olive oil production is a critical choice based on the best combination of oil quantity and oil quality. During ripening, in fact, a biochemical process occurs which causes both accumulation of oil and evolution of secondary plant metabolites. Oil accumulation in olive fruit, starting at the pit hardening stage, follows a curve whose course is affected by several factors, such as the cultivar and the cultural and environmental conditions.[1] Some of the most important aspects related to virgin olive oil quality are deeply affected by the olive ripening stage. The modification of the phenolic fraction, in particular, has been extensively investigated: the concentration of oleuropein reaches relatively high levels in immature fruit during the growth phase and declines with the physiological development of the fruit.[2,3] Because of the well-known importance of the phenolic fraction for oil stability and the sensory and health properties,[4] it is essential to identify the harvest period that ensures the ripening stage corresponding to the optimal phenolic content. Furthermore, the NDA Panel of the European Food Safety Authority (EFSA) concluded very recently that there is evidence of a cause and effect relationship between the consumption of olive oil polyphenols (standardized by the content of hydroxytyrosol and its derivatives) and the protection of LDL cholesterol particles from oxidative damage. The panel considers that in order to support the health claim, 5 mg of hydroxytyrosol and its derivatives in olive oil should be consumed daily, provided by moderate amounts of olive oil, warning that the concentrations in some olive oils may be too low to allow the consumption of this amount of polyphenols in the context of a balanced diet.[5]

Many approaches have been proposed in recent years for the evaluation of the optimal harvesting period, where efforts were aimed at finding a rapid prediction method based mainly on the oil content or indirect parameters.[6−9] The direct analysis of the qualitative and quantitative phenolic profile of olive fruit always includes an extraction step followed by chromatographic evaluation, becoming both costly and time-consuming.[10−15] Nowadays, the availability of a rapid, simple, objective and nondestructive method is highly desirable for the prediction of the phenolic content in olives during ripening.

Near-infrared spectroscopy (NIRS) can be considered an interesting, alternative technique for the nondestructive measurement of quality parameters in food crops, including fresh fruit and vegetables.[16,17] The NIR region contains

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information concerning the relative proportions of C−H, N−H, and O−H bonds, which are the primary structural components of organic molecules. This capability can be used as an alternative technique to the traditional destructive analytical methods usually used to define quality. Chemometry is required in the development of prediction models of qualitative attributes, and partial least-squares regression (PLSR)[18,19] is the statistical application usually employed to attain this goal.[20]

The use of NIRS on olive fruits and related products is already known; applications for the determination of oil and moisture content are now considered as routine analyses in comparison with relatively new methodologies, like NMR, or more traditional analytical determinations.[21,22] Leon et al.[23] tested NIRS, together with PLSR, carrying out prediction models for oil content, moisture, and fatty acid composition on olives intended for oil production. Results in terms of quality prediction of olive fruits by measuring moisture, dry matter, oil content, oil free acidity, and maturity index were obtained by Cayuela and del Carmen Pérez Camino,[24] working with a Vis/NIR Labspec, a spectral device equipped with three different detectors operating at specific wavelength ranges. Dupuy et al.[25] tested some different chemometric applications of NIR and mid-infrared (MIR) spectra, with the aim of predicting qualitative attributes and discriminating French cultivar origins using PLS discriminant analysis (PLS-DA). FT-IR (Fourier Transform-InfraRed) spectroscopy is another spectral technique already used for acidity estimation in intact olives[26] and for oil content and humidity in olive paste to monitor the oil process.[27] The NIR acousto-optically tunable filter (AOTF) is a spectral device whose high level of performance is mainly due to the wavelength selection capability of the acousto-optical filter and is described in detail by Barbieri-Gonzaga and Pasquini and by Workman and Burns.[28,29] Cayuela et al.[30] published a study in which the NIR-AOTF was used to predict fruit moisture, free acidity, and oil content in intact olives. The NIR-AOTF apparatus was tested very recently, coupled with the NMR technique, for the prediction of the % of oil content[31] in two Spanish olive cultivars. Jiménez et al.[32]applied NIR-AOTF and artificial neural network to develop a sensor software finalized to an optimization of the oil elaboration process. Other applications on different products are reported concerning NIR-AOTF spectroscopy: Kay and Wample[33] discriminated among areas of production of Cabernet Sauvignon grapes on the basis of total phenolics, anthocyanins, malvidin-3-glucoside, and tartaric and malic acid estimations; Santos and Kaye[34] applied the NIR-AOTF for potential grapevine leaf water detection. The same tool was tested by He et al.[35] for determination of principal constituents in tobacco, and more recently, Bellincontro et al[36−38] used NIR-AOTF spectroscopy to monitor grape dehydration and quality evolution of hazelnuts during storage. Phenolic content prediction by NIR spectroscopy was already tested in grapes[39] and in various food commodities,[40] but until now, there are no references in this field about olive fruits.

In this study, 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. The objective is to use the NIR-AOTF for field application to monitor ripening evolution on the basis of phenolic content. PLS models were developed for the prediction of total and specific phenols in olives for oil production (e.g., oleuropein, verbascoside, and 3,4-dihydroxyphenylethanol-elenolic acid). The reported results, even promising, refer only to a single year of production and need to be implemented and better validated. On this basis, the presented work can be considered an interesting study of feasibility with promising results which demonstrated the potentiality of the NIR-AOTF as on-field application for nondestructive evaluation of phenolic content in olives for oil production.

MATERIALS AND METHODS

Materials and Sampling Procedure. *Olives.* Drupes of Moraiolo, Dolce di Andria and Nocellara Etnea cultivars harvested in 2010 were used. The cultivar Moraiolo was chosen for its olives' high phenolic concentration content, whereas Dolce di Andria and Nocellara Etnea were chosen for their olives' low and medium phenolic content, respectively.

All the cultivars were grown in the germplasm collection of the Department of Agricultural and Environmental Sciences of the University of Perugia in the Umbria region (Central Italy; http://www.oleadb.it/collections/cultivar_coll_list.php?mastertable=collections&masterkey1=027) (43°04′54.58″N, 12°22′53.41″E). The collection is located on a hill at 320 m a.s.l.. The trees were 20 years old, trained according to the vase training system and spaced m 5 × 5. The orchard was rainfed. Fertilization was based on the supply of nitrogen, potassium, and phosphorus as chemical fertilizers. The soil was managed with a spontaneous green cover mowed 2−3 times/year. During the ripening period, olives were periodically collected for each cultivar from 3 labeled trees/cultivar (1 sample/tree). The sampling dates were September 23rd, October 14th, and November 10th, 2010, for all the cultivars considered. Olives were randomly collected around the equatorial part of the entire canopy. Shortly after collection, the samples were divided into 2 subsamples. Specifically, 6 sets of olive samples were collected at each sampling stage and sent for spectral detection; each sample set was represented by 5 (for cv. Nocellara Etnea) or10 (for cv. Moraiolo and Dolce di Andria) olives, with the number depending on the unitary fruit weight. This procedure was followed in order to ensure the correct amount of product required for the HPLC determinations, which were subsequently conducted.

Fruit Characteristics. The following fruit characteristics were determined: fresh weight (50 drupes/tree); pigmentation (50 drupes/tree) using the Ripening Index (Jaèn pigmentation index), ranging from 0 to 7, with 0 for green olives and 7 for olives with superficial pigmentation on 100% of the epicarp and 100% pigmentation on the pulp;[41] pulp (epicarp + mesocarp) firmness using a hand-held dynamometer with a 1.5 mm plunger (Effe.gi, Ravenna, Italy) (50 drupes/tree); water content by drying (at 105 °C) the samples used for fresh weight determination in an oven until constant weight; oil content (1 sample/tree) using the Foss-let 1531 apparatus (Foss Electric, Hilleröd, Denmark).

NIR Spectra Collection. A Luminar 5030 miniature Hand-held NIR Analyzer (Brimrose Corporation, Baltimore, 92 MD), based on the AOTF-NIR principle, was used for spectral detection.[30] This is a portable device which can be used directly in the field on-tree, even though in this specific case spectral detections were conducted under laboratory conditions. Two different measurements were performed on each intact olive through contact between the external gun of the NIR device and the epicarp of the fruit, using the diffuse reflectance method of detection, while the raw spectra were detected and recorded in transmittance, as reported by other authors.[34,35] Detection was conducted in the 1100−2300 nm range, with 2 nm wavelength increments and 10 spectra per average, which represented a single measurement. The average of the two measurements was the spectral response of the fruit.

Near Infrared Spectroscopy Analisys and Chemometrics. Raw spectra were statistically pretreated for absorbance (log $1/T$) transformation using SNAP 2.03 software (Brimrose). Before the calibration and building up of the prediction models, the spectral variations of the data sets were analyzed through Principal Component Analysis (PCA). The absorbance spectra, obtained as spectral average

Table 1. Fruit Characteristics of the Three Olive Cultivars Considered (Moraiolo, Dolce di Andria, and Nocellara Etnea) Evaluated at Three Different Ripening Stages[a]

cultivar	sampling date	fruit weight (g)	oil content (%/fw)	oil content (%/dw)	pulp firmness (N)	ripening index (0−7)
Moraiolo	23/09/2010	1.15 (0.02)	10.50 (1.00)	22.60 (1.40)	8.80 (0.10)	0 (0)
Moraiolo	14/10/2010	1.23 (0.03)	15.90 (0.70)	32.30 (0.90)	8.40 (0.09)	0.70 (0.10)
Moraiolo	10/11/2010	1.40 (0.02)	18.50 (0.90)	38.60 (0.80)	6.60 (0.16)	2.80 (0.20)
Dolce di Andria	23/09/2010	3.21 (0.09)	7.60 (0.30)	24.70 (1.00)	6.30 (0.35)	2.30 (0.30)
Dolce di Andria	14/10/2010	3.23 (0.17)	14.40 (1.40)	38.30 (2.40)	4.70 (0.16)	4.60 (0.10)
Dolce di Andria	10/11/2010	3.42 (0.08)	14.60 (1.20)	39.20 (1.90)	4.40 (0.12)	6.00 (0.10)
Nocellara Etnea	23/09/2010	2.01 (0.05)	9.40 (0.50)	20.00 (0.80)	8.50 (0.17)	0 (0)
Nocellara Etnea	14/10/2010	2.34 (0.08)	17.90 (0.50)	33.30 (0.70)	8.00 (0.12)	0.70 (0.20)
Nocellara Etnea	10/11/2010	2.76 (0.09)	21.10 (1.70)	42.10 (2.50)	5.50 (0.14)	1.90 (0.10)

[a]The values are the mean of three samples; the standard deviation is in parentheses.

of each olive subset, were used as X-variables for the final models. Mean normalization, Multiplicative Scattering Correction (MSC), and Standard Normal Variate (SNV) treatments, first order of the Savitzky-Golay filter (6 points of smoothing) or second order of the Savitzky-Golay filter (6 points of smoothing) were also tested, even though they were not used for the final modeling. In fact, absorbance spectra, without any other pretreatments, were identified as most effective in achieving the goal of the model calibrations. Partial Least Squares (PLS) models[19] were obtained on the full spectrum (1100−2300 nm), considering the spectral significant variables at specific wavelength intervals. The mean values and the ± SD values obtained by 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: oleuropein, verbascoside, 3-4 DHPEA-EDA, which were identified as the most relevant in quantity with respect to the other specific ones, and for total phenols, calculated as the sum of the measured compounds. Models were built for each olive cultivar by employing the total sample set of data (n = 54 in all cases, excluding Oleuropein in Dolce di Andria where n = 38), 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.[42] Models developed on the data set obtained as a combination of the three olive varieties were generated after division of the original data in two subsets: the largest of calibration (n = 115 in all cases, excluding oleuropein in Dolce di Andria where n = 110) and the smaller of prediction (n = 47 in all cases, excluding Oleuropein in Dolce di Andria where n = 36), which was employed as external validation. The generation of the data subsets was randomly obtained by SNAP 2.03. No outlier identification and elimination was applied. The statistical indexes R^2 (coefficient of multiple determination) in calibration, cross-validation, and prediction ; Root Mean Standard Error of Calibration (RMSEC), Root Mean Standard Error of Cross-Validation (RMSECV), Root Mean Standard Error of Prediction (RMSEP), and bias were used to determine the significance of the calculations. RPD values, defined as the ratio between SD and SECV[443], were also calculated for all prediction models carried out. PCA, statistical pretreatments, and PLS models were performed using Unscrambler v9.7 software (CAMO ASA, Oslo, Norway); graphs, score plot, and scatter plots were performed, after data exportation from Unscambler, using SigmaPlot v. 11.0 (Systat Software Inc., San Jose, CA, USA).

Reference Compounds. (3,4-Dihydroxyphenyl)ethanol (3,4-DHPEA) was obtained from Cabru (Milan, Italy), while (*p*-hydroxyphenyl)ethanol (p-HPEA) was obtained from Sigma Aldrich (Milan, Italy). Oleuropein glucoside was purchased from Extrasynthèse (France). Demethyloleuropein and verbascoside were extracted from olive fruit according to the procedure reported in a previous paper.[44] The dialdehydic forms of elenolic acid linked to 3,4-DHPEA (3,4-DHPEA-EDA), (+)-1-acetoxypinoresinol, and (+)-pinoresinol were extracted from VOO using a previously reported procedure.[45] The purity of all the extracted substances was tested by HPLC, and their chemical structures were verified by NMR using the same operational conditions reported in previous papers[44,46]

Extraction and HPLC Analysis to Obtain Reference Compounds. The same fruits employed for spectral detection were frozen in liquid nitrogen, stored at −80 °C, and successively used for phenolic determination. The phenols were extracted from the olive pulp according to the procedure published previously by Servili et al.,[47] modified as follows: 5 g of frozen olive pulp was homogenized with 100 mL of 80% methanol containing 20 mg/L of sodium diethyl dithiocarbamate; the extraction was performed in triplicate. After methanol removal, the aqueous extract was used for the extraction by solid-phase separation (SPE) of phenols. The SPE procedure was applied, for the phenolic extracts, by loading a 900 mg Maxi-Clean high load C18 cartridge (Alltech Italia S.r.l., Sedriano, Italy) with 1 mL of sample, using 50 mL of methanol as the eluting solvent. After solvent removal under vacuum at 30 °C, the phenolic extract was recovered and then dissolved in methanol (1 mL). The reversed phase HPLC analyses of phenolic extracts were conducted with an Agilent Technologies system model 1100 composed of a vacuum degasser, a quaternary pump, an autosampler, a thermostatted column compartment, a DAD, and a fluorescence detector (FLD). For the evaluation of the phenolic compounds,[48] a Spherisorb ODS-1 column (250 × 4.6 mm with a particle size of 5 μm, phase Separation Ltd., Deeside, UK) was used, and a 20 μL sample volume was injected. Lignans were detected by an FLD operated at an excitation wavelength of 280 nm and emission at 339 nm,[45] while the other compounds were detected by DAD at 278 nm.

Statistical Analysis. The averaged results of the triplicate HPLC detections were statistically tested by ANOVA. Tukey's test was applied in order to establish significant differences ($P < 0.05$), which were graphically marked by letter assignments. Possible intercorrelations among specific and total phenols were evaluated by determination of the Pearson's coefficients.

RESULTS AND DISCUSSION

In the olive ripening period taken into consideration, fruit weight was similar in stages I and II and increased from stage II to stage III in all the examined cultivars (Table 1). The pigmentation and the pulp firmness of the fruits increased and decreased, respectively, throughout the considered period, with Dolce di Andria showing the highest pigmentation values (with significant pigmentation also at stage I) and the lowest firmness values (Table 1). All the cultivars showed continuous accumulation of oil in the drupes. In general, during ripening, the characteristics of the fruits of the cultivars Moraiolo and Dolce di Andria can be considered to be in agreement with those reported in the literature for these varieties (see Web site www.oleadb.it), whereas Nocella Etnea had a relatively low fruit weight, probably because of a very high fruit load on the trees, which was also associated with relatively low amounts of rainfall.

However, relatively low values for this cultivar grown in the Umbria region were also observed in another study.[49] The

phenolic composition did not show significant qualitative modifications during ripening, the only exception being the presence of demethyloleuropein in Nocellara and the disappearance of oleuropein in Dolce di Andria, both observed between the IInd and the last stage of ripening (Table 2). In Moraiolo and Nocellara olives, the total phenolic content decreased significantly according to the ripening stage, mainly due to the reduction in 3,4-DHPEA-EDA, oleuropein, and verbascoside content. A slight increase in pinoresinol was observed in Nocellara also. On the contrary, in the olives from Dolce di Andria, the total content and the number of single compounds remained constant for the entire period studied. These results are in agreement with previous studies that show a strong cultivar impact in the qualitative and quantitative evolution of secoiridoids and verbascoside in olive fruit during ripening.[2,50]

On the data set of all phenols (specific and total) used for PLS modeling, possible effect of intercorrelations were evaluated by correlation matrix and Pearson's coefficients (data not shown). Results demonstrated non-negative correlations among specific and total phenols which were proportional to the influence of each single compound on the total amount (3,4-DHPEA-EDA > verbascoside > oleuropeina). This evidence is, in some ways, obvious if we think that we are talking about numeric variables and the total content of phenols was calculated as the sum of the measured specific compounds. In any case, it is necessary to consider that, as a consequence of the intercorrelation among dependent variables, an effect of subrogation could occur, and this phenomenon is potentially dangerous in the practical application of predicting models.

In Figure 1A, the absorbance mean spectra relative to all the acquisitions for each single varietal group of olives are reported. Even without considering specific differences in terms of the height of the peaks observed among the cultivars (the NIR spectra appear to be similar for all samples), we can describe the most significant band correlation. A first band, with a very small absorbance, is observed at 1150 nm and can be considered as a combination of the symmetric and asymmetric OH stretching and OH bending bands. A second band is observed at 1200 nm and corresponds to the second overtone of the CH stretching vibrations of CH_3, CH_2, and CH═CH. Spectra are characterized by two principal water absorption bands of around 1450 nm and 1920−1950 nm.[51] They are assigned 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 (1920−1950 nm), respectively.[52,53] The two bands located at 1720 and 1750 nm correspond to the first overtone of the CH stretching vibrations of CH_3, CH_2 and CH═CH. The last band in these typical olive fruit spectra was observed at 2250 nm and is due to the combination of the CH stretching vibrations of CH_3, CH_2 with other vibrations. The peaks observed at 1200, 1720−1750, and 2250 nm are typically attributed by the references to the presence of oil,[25] which was not considered in this study in terms of estimated parameter. Instead, no references or previous studies are available on phenolic detections by near-infrared spectroscopy on olive fruits, and thus, no correlation bands are known in this regard.

In a very recent study directed at NIR application for phenolic determination on grape skins, Ferrer-Gallego et al.[39] identified in the spectral region in the range from 1140 to 1320 nm a relevant contribution to the loadings of their models. In our case, we could suggest spectral correlations related to the

Table 2. Phenolic Composition (mg/g of Fresh Weight) of Olive Fruits from Moraiolo cv. Evaluated at Three Different Ripening Stages[a]

	cv. Moraiolo			cv. Dolce di Andria			cv. Nocellara		
compds	I stage	II stage	III stage	I stage	II stage	III stage	I stage	II stage	III stage
3,4-DHPEA[b]	0.50 (0.06)a	0.50 (0.05)a	0.50 (0.04)a	0.10 (0.05)a	0.20 (0.05)a	0.10 (0.004)a	0.30 (0.04)a	0.30 (0.03)a	0.30 (0.07)a
p-HPEA	0.05 (0.01)a	0.04 (0.05)a	0.04 (0.03)a	0.10 (0.02)a	0.10 (0.01)a	0.10 (0.03)a	0.30 (0.06)a	0.20 (0.02)ab	0.10 (0.03)b
demethyloleuropein	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2.4 (0.40)
verbascoside	10.70 (0.70)a	5.60 (0.30)b	3.90 (0.20)c	1.60 (0.40)a	1.30 (0.20)a	1.20 (0.30)a	23.70 (0.60)a	11.20 (0.20)b	10.40 (0.10)b
3-4 DHPEA-EDA	23.20 (0.90)a	16.90 (0.80)b	13.20 (0.80)c	1.80 (0.40)a	1.40 (0.60)a	1.00 (0.20)a	21.50 (0.70)a	15.40 (0.30)b	10.90 (0.20)c
oleuropein	6.60 (0.90)a	3.60 (0.40)b	3.70 (0.60)b	0.80 (0.30)a	0.40 (0.10)a	n.d.	3.30 (0.50)a	2.40 (0.10)b	2.40 (0.04)b
(+)-1-acetoxypinoresinol	0.50 (0.30)a	0.40 (0.10)a	0.40 (0.08)a	0.30 (0.08)a	0.30 (0.02)a	0.30 (0.06)a	0.40 (0.20)a	0.40 (0.06)a	0.30 (0.03)a
(+)-pinoresinol	0.30 (0.02)a	0.3 (0.01)a	0.20 (0.02)a	0.20 (0.04)a	0.20 (0.06)a	0.20 (0.07)a	0.20 (0.01)a	0.30 (0.03)a	0.50 (0.07)b
sum of phenols	41.90 (1.90)a	27.40 (1.30)b	22.00 (1.30)c	4.80 (0.90)a	3.80 (0.90)a	2.90 (0.50)a	49.80 (1.40)a	30.20 (0.60)b	27.30 (0.60)c

[a]Values in the same row with different letters differ significantly ($p < 0.05$). [b]The phenolic content is reported as the mean value of six samples analyzed in duplicate; the standard deviation is in parentheses.

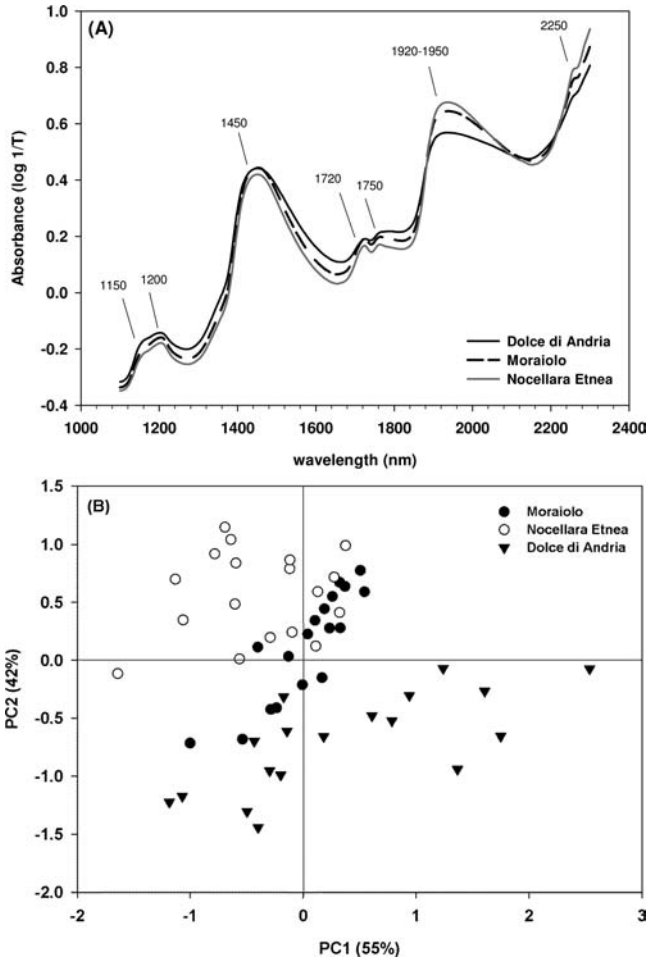

Figure 1. (A) NIR-AOTF mean spectra of all olive samples measured during their ripening evolution. Spectra are reported as absorbance units calculated from the original detections (log 1/T) and plotted versus the wavelengths (nm) where significant bands were pointed out. (B) Score plot of the principal component analysis (PC1 vs PC2) carried out on the absorbance NIR-AOTF spectra of grouped samples coming from all three olive cultivars (Moraiolo, Dolce di Andria, and Nocellara Etnea). Percent of the explained variance is reported in parentheses on the axes.

peaks identified at 1150 and 1200 nm, respectively, described previously. However, this suggestion seems to be confirmed by the results reported in Figure 2, where PLS regression coefficients for the total phenol model were plotted versus wavelengths. At the same time, other authors have reported a possible correlation between phenols and tannins in correspondence to the 1650−1750 nm region;[54] on the basis of this suggestion, we may consider a contribution due to the band correlation identified at 1720 and 1750 nm and partially confirmed by the same PLS regression coefficient plot (Figure 2).

More generally, the results observed in this graph suggest that in PLS modeling for phenolic prediction by NIR application, many spectral contributions were found at many wavelengths, defining a combined effect in this spectral detection range (1100−2300) with the performance of the NIR-AOTF device. In PLS modeling for phenol calibrations and predictions, specific wavelengths were not selected, and the entire spectrum (1100−2300 nm) was considered.

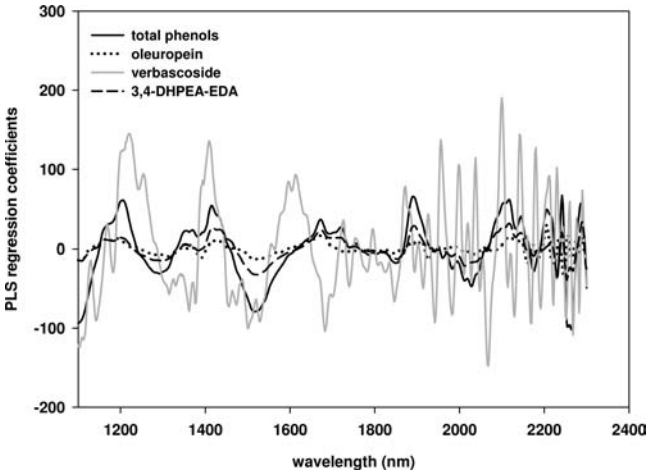

Figure 2. PLS regression coefficients obtained for the prediction model of total phenols carried out on each single cultivar and on the global data set of olive samples (sum of the three cultivars). Data are plotted versus the wavelengths (nm).

Preliminary PCA, carried out on all the spectral detections, was used just for sample description, while outlier selection was not applied. The results from the same PCA (Figure 1B) demonstrate that the totality of the variance is practically explained by PC1 and PC2 (55% and 42%, respectively), while 4 PCs were required for the explanation of total residual variance (99%).

In terms of sample distribution, an appreciable separation among all samples referring to the three cultivars used was obtained with, in detail, a good discrimination among samples of the cultivar Dolce di Andria and the two other cultivars, while the separation between samples of the cultivars Moraiolo and Nocellara Etneaappears was a bit difficult.

In all chemometric approaches, the variability in the concentration of the parameters, measured by destructive detections, is relevant for modeling. In Table 3, analytical contents with respect to the total polyphenols and the three specific phenols (verbascoside, oleuropein, and 3,4 DHPEA-EDA) included in this study were statistically defined by descriptive indexes, which include range (as min and max values), mean, and SD. Reported data describe well how the three stages of sampling allowed obtaining a favorable variability of the data sets for all specific and total phenols studied and used for the multivariate calibration models. For regression models, as reported in the Materials and Methods section, different pretreatments were performed on the spectra sets (data not shown), but in the end, raw spectra only transformed in absorbance (log $1/T$) proved to be the best performing and were used in subsequent chemometric applications.

Calibration and cross-validation results for all models, in terms of estimated phenolics and olive varieties, are reported in Table 4. Characteristic scatter plots, clearly depicting the same results graphically, were included for the 4 models carried out for the sum of the three varieties (global models), which were obtained on the basis of a calibration set of data (validated by cross-validation) and validated by external data (Figure 3A−D). The complete chemometric results are reported in Table 5.

Promising results in terms of correlation were obtained for all the models in all cases of tested olive samples (cultivars), including global models carried out as the sum of three varieties

Table 3. Statistical Analyses of Sample Sets Relative to Each Cultivar (Moraiolo, Dolce di Andria, and Nocellara Etnea) and to Their Sum[a]

		cv. Moraiolo (180 drupes)			
		total phenols	verbascoside	oleuropein	3,4 DHPEA-EDA
data set (*n*)		54	54	54	54
mean		30.39	6.71	4.66	17.77
SD		1.54	3.06	1.53	4.46
min		17.90	2.90	2.57	8.30
max		44.40	12.20	7.60	25.50
		cv. Dolce di Andria (180 drupes)			
		total phenols	verbascoside	oleuropein	3,4 DHPEA-EDA
data set (*n*)		54	54	38	54
mean		3.84	1.38	0.59	1.39
SD		1.27	1.00	0.32	0.47
min		2.10	0	0.20	0.50
max		7.60	4.20	1.40	2.60
		cv. Nocellara Etnea (90 drupes)			
		total phenols	verbascoside	oleuropein	3,4 DHPEA-EDA
data set (*n*)		54	54	54	54
mean		35.76	15.12	2.70	15.93
SD		15.22	9.46	1.39	5.24
min		16.54	3.16	0.96	8.24
max		57.07	28.08	5.32	23.69
		sum of the three varieties (450 drupes)			
		total phenols	verbascoside	oleuropein	3,4 DHPEA-EDA
data set (*n*)	C	115	115	110	115
	P	47	47	36	47
mean	C	22.81	7.88	2.85	11.01
	P	24.59	7.35	2.93	13.35
SD	C	16.99	8.27	1.97	8.51
	P	18.04	7.59	2.24	7.75
min	C	2.09	0.10	0.20	0.50
	P	2.50	0.10	0.20	0.60
max	C	57.07	28.08	7.59	25.50
	P	56.30	24.71	7.19	24.50

[a]Mean, standard deviation (SD), range (min and max) for total phenols, verbascoside, oleuropein, and DHPEA-EDA were reported expressed as mg/g of fresh weight. Data relative to the sum of three varieties and are separated into two different subsets: calibration (C) and prediction (P).

used and validated by a separated subset of data. In PLS models built for the cultivar Dolce di Andria, the R^2 (coefficients of determination) in calibration ranged between 0.93 and 0.98, while the R^2 in cross-validation ranged between 0.837 and 0.956; for the cultivar Moraiolo, the indexes ranged between 0.948 and 0.975, 0.934, and 0.966, respectively; for the cultivar Nocellara Etnea, between 0.993 and 0.998 for R^2 in calibration, and 0.98 and 0.992 for R^2in cross-validation. Generally speaking, for the estimation of the predictive accuracy of the models, an R^2cv (coefficients of determination in cross-validation) greater than 0.9 represents a valid quantitative information.[52] Excluding the results obtained in PLS models built for oleuropein and 3,4 DHPEA-EDA in the case of the cultivar Dolce di Andria (0.837 and 0.862, respectively), this goal was achieved in all of the other cases presented in this study (Table 4).

The literature reports that cross-validation is a practical method for demonstrating how NIRS can predict a qualitative attribute, even if it would be better to estimate the accuracy of the application by using an appropriate, preferably external, test, or validation set.[42] However, in leave-one-out cross-validation, one sample is removed from the data set, and a calibration model is built on the basis of the remaining subset. Removed samples are then used to calculate the prediction residual.[20] The process is completed when every sample has been left out once, and in the end, the variance of all prediction residuals is estimated. For all practical purposes, this validation method can be considered satisfactory when the experimental data set is limited, and it is not possible to arrange it in two separate subsets: one, the biggest, for the calibration, and the other for the external validation.

In the specificity of this context, we have to consider that, starting from a total of 450 drupes sampled and 900 spectral measurements performed, we only obtained a maximum of 162 reference values of specific total phenols (Table 3). This is due to the need to group the olive fruits in order to obtain the extractions for the subsequent HPLC analyses which, at the same time, were long laboratory procedures. In any case, a separated subset of data were arranged in the case of the larger sets of data (sum of three varieties) and the external predictions were performed. In Table 5, it is possible to observe the obtained R^2 in calibration which were of 0.925 (total phenols), 0.958 (3,4-DHPEA-EDA), 0.961 (oleuropein), and 0.972 (verbascoside), while the coefficients of determination in cross-validation were equal to 0.896 (total phenols), 0.925 (oleuropein), 0.926 (verbascoside), and 0.943 (3,4-DHPEA-EDA).

Finally, external validations allow R^2 in the prediction of 0.874 fot total phenols, 0.903 for oleuropein, 0.924 for 3,4-DHPEA-EDA, and 0.942 in the case of verbascoside. Significant results obtained in terms of correlation on the predictive models can also be attributed to the high degree of accuracy and precision of the reference data measured using the HPLC method. The importance, in chemometry, of the accuracy in estimating the destructive data has already been considered and pointed out.[20] However, the real and applicative performance of the predictive models is better defined if combined with the estimation indexes of the obtainable potential errors in calibration and prediction or cross-validation (RMSEC, RMSEP, and RMSECV). Results obtained for RMSECV (expressed as mg/g of olive fresh weight) are promising in expressing the predictive performance of the models. They ranged between 0.175 and 1.672 in all the models carried out individually for all three olive cultivars (Table 4) and rose up to 5.48 in the case of the total phenol estimation considering the global model obtained by the sum of the three varieties (Table 5). In Table 5 are also reported RMSEP indexes (mg/g) which were equal to 0.68 for oleuropein, 1.79 for verbascoside, 2.1 for 3,4-DHPEA-EDA, and 6.33 for total phenols.

In calibration developments of all developed models, the number of latent variables (LVs) was calculated and selected in correspondence to the RMSECV minimization; they are reported in Table 4. A very large variability among results in LVs is observable (from a minimum of 2 up to a maximum of 13) and that is certainly influenced by the complexity of the independent variables (X-block) involved in PLS regression.[55] A larger number of samples could reduce the number of LVs and, consequently, possible errors in predictive responses of PLS models.[20]

The RPD values for all the models in all cases tested are also reported in Table 4. The RPD ratio is another statistical index

Table 4. Calibration and Cross-Validation Results in PLS Models for total Phenols, verbascoside, oleuropein, and DHPEA-EDA Calculated on the Data Sets Coming from the Three Different Cultivars (Moraiolo, Dolce di Andria, and Nocellara Etnea)[a]

	calibration				cross-validation		
	R^2	RMSEC	Bias	LVs	R^2	RMSECV	RPD
			Moraiolo				
total phenols n = 54	0.974	1.378	-5.30×10^{-7}	5	0.965	1.631	1.860
verbascoside (n = 54)	0.948	0.727	-3.97×10^{-8}	2	0.934	0.774	1.960
oleuropein (n = 54)	0.973	0.262	-1.41×10^{-6}	9	0.947	0.365	4.180
3,4 DHPEA-EDA (n = 54)	0.975	0.980	-1.77×10^{-8}	5	0.966	1.138	3.890
			Dolce di Andria				
total phenols (n = 54)	0.943	0.298	-4.20×10^{-6}	13	0.904	0.451	2.780
verbascoside (n = 54)	0.980	0.137	-4.63×10^{-6}	13	0.956	0.210	4.720
oleuropein (n = 38)	0.930	0.114	-1.22×10^{-7}	9	0.837	0.175	1.800
3,4 DHPEA-EDA (n = 54)	0.938	0.159	1.44×10^{-6}	13	0.862	0.237	1.940
			Nocellara Etnea				
total phenols n = 54	0.993	1.216	7.06×10^{-8}	8	0.988	1.672	9.020
verbascoside (n = 54)	0.993	0.760	2.01×10^{-6}	7	0.985	1.000	9.360
oleuropein (n = 54)	0.990	0.132	-1.87×10^{-7}	10	0.980	0.195	7.060
3,4 DHPEA-EDA (n = 54)	0.998	0.257	4.40×10^{-6}	11	0.992	0.473	10.970

[a]RMSEC and RMSECV are expressed as mg/g of fresh weight.

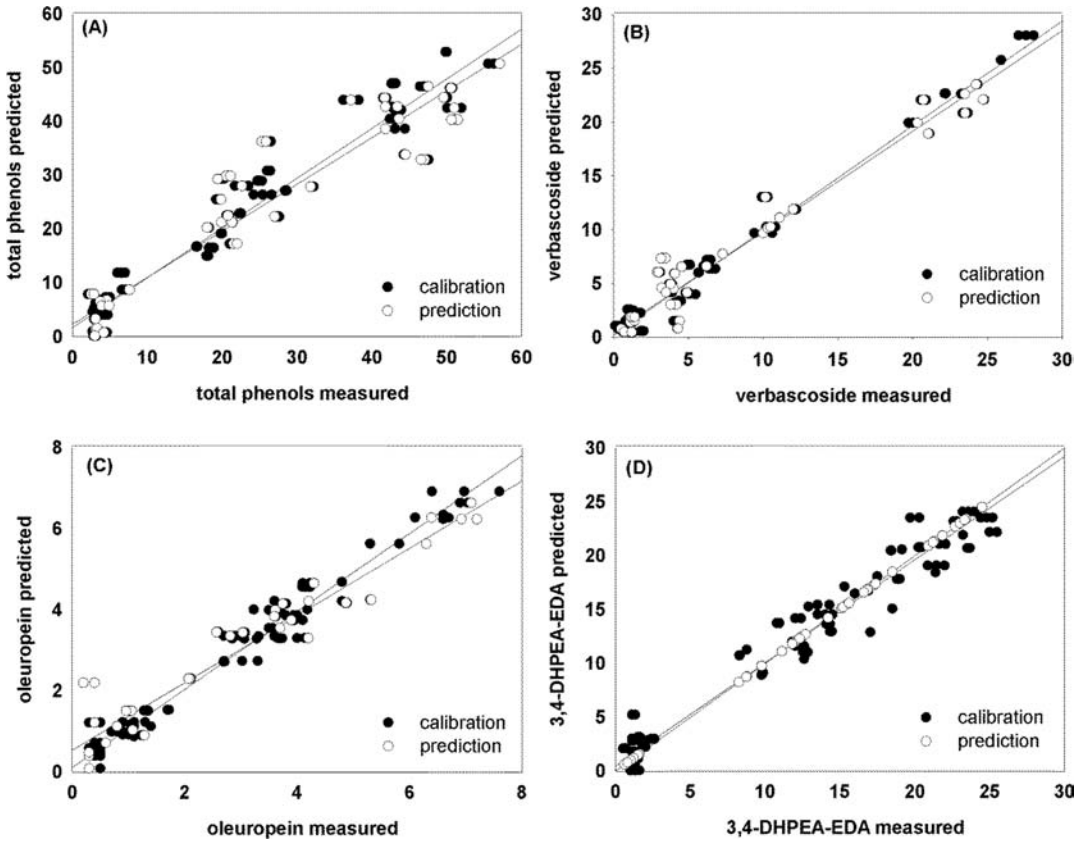

Figure 3. Scatter plots relative to the prediction models for DHPEA-EDA (a), verbascoside (b), oleuropein (c), and total phenols (d) carried out on the global data set of olive samples (sum of the three cultivars). For each compound measured, values are plotted versus predicted values, and calibration and validation data sets are reported.

useful for evaluating the predictive ability of the NIRS: an RPD below 2.5−3 means that the model can discriminate low from high values of the response variable;[43,56] values higher than 5 indicate good discrimination, especially if destined for quality and food control.[43,57] RPD ratios that were just barely insufficient were obtained for oleuropein and 3,4 DHPEA-EDA models in the cultivar Dolce di Andria (1.8 and 1.94, respectively), and for total phenols and verbascoside models in the cultivar Moraiolo (1.86 and 1.96, respectively). All other RPD ratios were completely sufficient, within the estimation range over 3, or describing a great capacity, as previously described, when the values were greater than 5, as in the case of the cultivar Nocellara Etnea when the RPD values were 9.02, 9.36, 7.06, and 10.97 for total phenols, verbascoside, oleuropein, and 3,4-DHPEA-EDA, respectively. RPD ratios related to the models carried out on the total set of data and

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Table 5. Calibration, Cross-Validation, and Prediction Results in PLS Models for total Phenols, verbascoside, oleuropein, and DHPEA-EDA Calculated on the Data Sets Obtained as a Combination of the Three Different Cultivars (Moraiolo, Dolce di Andria, and Nocellara Etnea)[a]

	calibration					cross-validation			prediction		
	n	R^2	RMSEC	bias	LVs	R^2	RMSECV	RPD	n	R^2	RMSEP
					sum of three varieties						
total phenols	115	0.925	4.610	3.00×10^{-5}	9	0.896	5.480	3.100	47	0.874	6.330
verbascoside	115	0.972	1.370	2.67×10^{-5}	13	0.926	2.250	3.670	47	0.942	1.790
oleuropein	110	0.961	0.380	2.50×10^{-6}	12	0.925	0.540	3.640	36	0.903	0.680
3,4 DHPEA-EDA	115	0.958	1.730	-7.31×10^{-8}	9	0.943	2.050	4.150	47	0.924	2.100

[a]RMSEC, RMSECV, and RMSEP are expressed as mg/g of fresh weight.

external validated (Table 5) ranged between 3.10 (total phenols) and 4.15 (3,4-DHPEA-EDA).

In the end, all results reported in this study, as in terms of correlations as well of validation, are quite promising in demonstrating the feasibility of the NIR-AOTF application intended for the modeling for the prediction of total and characteristic phenols on intact olive fruits for oil production. Certainly, the addition of the number of samples to the predicting models could improve their robustness; also, the sample collection in different years of production could extend their biological variability, and we are working toward this.

In conclusion, the definition of the optimal ripening stage of olive fruit is a strategic point for producing high quality virgin olive oil. In this context, phenolic compounds are the most important substances that define the quality of virgin olive; thus, they can be considered an important analytical marker for indicating the best ripening stage of fruit in combination with traditional indexes such as oil accumulation. This is the first article to report results that define a rapid method for evaluating phenolic compounds directly in olives using nondestructive technology such as NIR-AOTF spectroscopy, which presents the important advantage that it can be used on-field, giving promising results even for measuring oil accumulation in olive fruits.

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Notes

The authors declare no competing financial interest.

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