JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Parent and Harvest Year Effects on Near-Infrared Reflectance Spectroscopic Analysis of Olive (*Olea europaea* L.) Fruit Traits

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The influence of parent and harvest year on the determination of oil, moisture, oleic acid, and linoleic acid contents in intact olive fruit was studied by near-infrared spectroscopy (NIRS). Spectral data from 400 to 1700 nm were recorded on 437 fruit samples collected in 1996 and 1997 from seedling plants derived from three different female parents. Partial least squares models were developed using samples for each year and for each female parent separately and were validated against the other groups. Calibration models were accurate enough to predict all constituents in new samples from a different female parent but were not transferable across years. However, a calibration equation of sufficient accuracy was obtained from the combined data set (*r* values of 0.94, 0.93, 0.84, and 0.88 and RMSECV values of 1.33, 1.88, 4.73, and 2.91 for oil, moisture, oleic acid, and linoleic acid contents, respectively). These results demonstrate the utility of NIRS as a selection tool in olive breeding programs.

KEYWORDS: Fatty acid composition; intact fruits; NIRS; oil content; *Olea europaea*

INTRODUCTION

In 1992, an olive breeding program was initiated in Córdoba (Spain) aimed at obtaining new olive cultivars with some of the following traits: early bearing, high yield and oil content, resistance to peacock eye (*Spilocaea oleagina*, Cast), suitability for mechanical harvesting, and high olive oil quality (*1*). Many samples must be analyzed in breeding programs, so there is a need for fast and cheap analytical procedures to determine the agronomical traits of interest. The Soxhlet is the official method to determine the oil content of olive fruit, and the fatty acid composition of olive oil is usually determined by gas chromatography of the corresponding methyl esters (*2*). These methods require too much time and are impractical for processing large numbers of samples so that new procedures have been developed to simplify these determinations. Nuclear magnetic resonance (NMR) has widely substituted the Soxhlet procedure. NMR analysis requires less sample manipulation and time and allows the use of whole olives, although samples still have to be dried until constant mass before analysis (*3*). Some new procedures to prepare fatty acid methyl esters directly from fresh tissues have also been developed, permitting the analysis of fatty acids without previous lipid extraction (*4*). These methods, however, are still costly and time-consuming and require the use of chemical reagents and considerable sample manipulation, especially for large sample sets. As an alternative, near-infrared spectroscopy (NIRS) offers many advantages such as the rapid and simultaneous nondestructive analysis of many traits with low analytical cost per sample and without the use of chemical reagents. The usefulness of NIRS as a tool in breeding programs has been mentioned from the beginnings of the development of this technology (*5*), so that NIRS has been and is being used to assist plant breeders in the selection of superior genotypes in breeding programs (*6*).

Previous results demonstrated that NIR spectra from intact olive fruits can be used to accurately predict oil and oleic acid content, two of the most important traits in olive breeding programs (*7*). However, the study was undertaken using a closed population of samples collected in the same year and there was little information available regarding the robustness of calibration models developed in the previous work for the prediction of independent sample populations. Calibration sets must be representative of the biological variability of future samples in order to obtain accurate predictions for these samples. To develop robust multivariate calibration models, therefore, calibration data must be obtained over a sufficient period of time to span an appropriate range of instrumental and environmental conditions covering the concentration of the traits of interest as well as other biological factors (*8*, *9*).

Several studies have shown the influence of genetic and environmental factors on the characteristics of olive oil, especially in relation to fatty acid composition. Although the influence of cultivar seems to be predominant, other factors such as year of harvest, location, and climatic conditions could also have a great influence on the variation of some fatty acids (*10*−*13*). In addition, the evaluation of olive progenies previously

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10.1021/jf0496853 CCC: $27.50
Published on Web 07/08/2004

reported in other olive breeding programs is rather complex because some characteristics such as oil content seem to stabilize only after 2−3 years (*14*).

The objective of this work is to confirm the usefulness of NIRS analysis of intact olive fruit as a selection tool in olive breeding programs. For this purpose, we must demonstrate that calibrations developed to estimate the constituents of interest are transferable across different sample populations. In this work, the performance of NIRS calibrations to estimate moisture, oil content, oleic acid, and linoleic acid in frozen intact olive fruits is evaluated in fruit samples from seedling plants derived from different female parents and collected in different years.

MATERIALS AND METHODS

Samples and Reference Methods. Seedlings from crosses between Arbequina, Frantoio, and Picual olive cultivars were used in this study. Parents of the breeding program were chosen on the basis of their high yield and oil content, different geographical origin, and differences in precocity of first bearing and fatty acid composition (*1*). Seedlings were transplanted into the field in 1994 at distances of 3.50 m between rows and 1.50 m between trees in the row. Standard cultural practices were followed in the orchard located in Cordoba (37° 51′ 42″ N, 04° 48′ 00″ W) to ensure tree growth. Fruit traits were evaluated in 1996 and 1997 once seedlings began to flower and produce fruit.

Three samples, each of 50 olives, were prepared to obtain reference oil and moisture content data. Fresh samples were dried in a forced-air oven at 105 °C for 42 h, and the oil content of dried samples was recorded by NMR (*3*). Fatty acid methyl esters (FAMEs) were prepared following the procedure of Garcés and Mancha (*4*) and separated using a flame ionization detector gas chromatograph equipped with a BPX-70 column (50 m, 0.25 mm i.d., 0.22 mm film thickness). Ten samples per seedling (each for a single fruit) were analyzed, and two fatty acids, oleic and linoleic, expressed as a percentage of total fatty acids, were monitored in this study. Fatty acids were determined in 147 and 287 seedlings in 1996 and 1997, respectively. Oil and moisture contents were measured only in 96 and 224 seedlings in 1996 and 1997, respectively, because of the higher amount of fruit necessary for these analyses.

Near-Infrared Spectroscopy and Chemometrics. Spectra were obtained using a DA-7000 diode array VIS/NIR analysis system (Perten Instruments, Huddinge, Sweden). Intact frozen olive fruit samples were scanned in reflectance mode over the range 400−1700 nm with a spectral resolution of 5 nm. Data collection was done using Perten DA7000 software (Simplicity), and data analysis was carried out using The Unscrambler (CAMO A/S, Trondheim, Norway).

Principal component analysis (PCA) was used to investigate sample spectra, and calibration models were developed and evaluated using partial least squares (PLS) regression. Data analysis was carried out using the wavelength range from 900 to 1500 nm after pretreatments by calculation of Savitzky−Golay second derivatives (5 data points gap size). None of the other mathematical pretreatments (multiplicative scatter correction, first derivative) or wavelength ranges tested improved the prediction accuracy of the PLS models. Calibration models were developed for each year and for each female parent separately within the second year data and validated against the other groups (the other year and the other female parents, respectively). Full cross-validation (i.e., leave-one-out) was used for determining the performance of the models within each group. External validation with the other groups was used to assess the performance of calibrations across different populations of olive fruit samples (different years or female parents). Correlation between actual and predicted constituent values (r), bias, standard error of cross validation (RMSECV), and standard error of prediction (RMSEP) on a separate sample set were used to test the performance of calibrations (*15*). The range error ratio (RER), defined as the ratio between the data set range for any given constituent and the standard error of cross validation or prediction for the same constituent, was also determined to indicate the relative utility of each model (*16*).

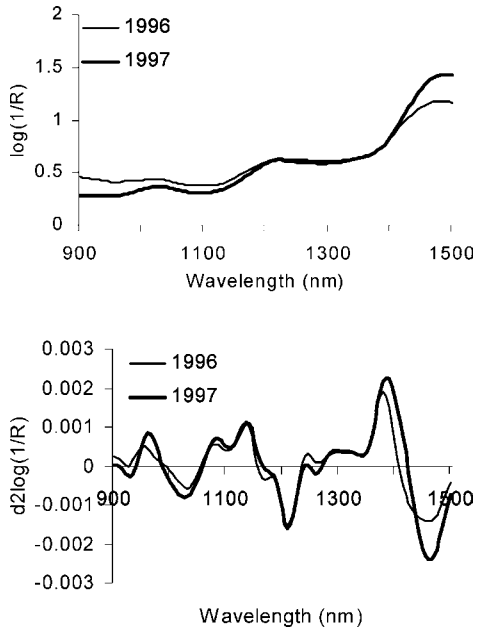

Figure 1. Average raw and second derivative spectra of olive fruit samples by year.

Table 1. Descriptive Statistics for Oil, Moisture, Oleic Acid, and Linoleic Acid Contents by Year and by Female Parent within the Second Year

constituent	group	*N*	mean	SD	min	max
oil[a]	1996	96	18.3	3.7	7.7	28.9
	1997	224	14.2	3.1	5.9	22.9
	female Arbequina	116	13.7	2.9	6.1	21.8
	female Frantoio	31	15.3	3.3	9.7	22.5
	female Picual	77	14.6	3.3	5.9	22.9
moisture[a]	1996	96	62.0	4.7	45.5	79.1
	1997	224	68.7	3.8	57.0	78.7
	female Arbequina	116	69.2	3.6	58.9	78.7
	female Frantoio	31	68.1	3.6	60.8	74.5
	female Picual	77	68.1	4.1	57.0	77.1
oleic acid[b]	1996	147	71.3	6.7	50.9	82.7
	1997	287	65.7	9.1	43.6	84.7
	female Arbequina	147	64.5	9.5	43.5	84.6
	female Frantoio	45	68.2	6.9	50.0	79.7
	female Picual	95	66.2	9.0	46.5	84.7
linoleic acid[b]	1996	147	8.0	4.5	1.6	22.3
	1997	287	11.6	6.4	1.6	29.2
	female Arbequina	147	12.1	6.7	1.6	29.2
	female Frantoio	45	10.5	5.7	3.5	23.2
	female Picual	95	11.4	6.2	1.7	25.0

[a] % w/w. [b] % of total fatty acids.

RESULTS AND DISCUSSION

Descriptive statistics for oil, moisture, oleic acid, and linoleic acid contents by year and female parent are shown in **Table 1**. Progenies showed a wide range of variation for all the characters evaluated in both years and irrespective of the female parent considered with values ranging from 5.9 to 28.9% for oil content, from 45.5 to 79.1% for moisture, from 43.6 to 84.7% of total fatty acids for oleic acid, and from 1.6 to 29.2% of total fatty acids for linoleic acid. The range of variation was as large or even slightly larger than that reported in olive cultivar collections (*12*, *17*) and similar to reports of wild populations (*18*).

Spectra. Original (log(1/R)) and second derivative (d^2 log(1/R)) average spectra of olives by year and female parent are shown in **Figure 1**. The shape of the original spectra was relatively flat, with the dominant feature being the peak around

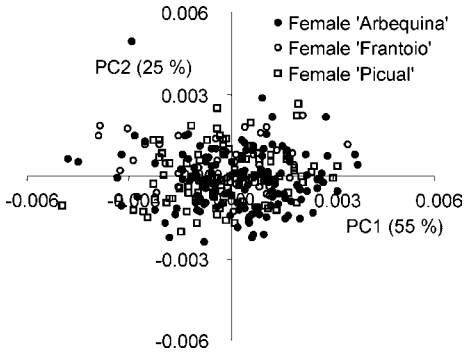

Figure 2. Biplot of scores on principal components 1 and 2 of samples analyzed for fatty acids in 1997.

Table 2. Cross-Validation (CV) and External Validation Results for Arbequina Female Calibration Models Developed Using Samples Collected in 1997

constituent	validation group	*r*	RMSECV	RMSEP	bias
oil[a]	CV	0.96	0.87		0.01
	female Frantoio	0.97		0.84	–0.13
	female Picual	0.96		0.94	–0.15
moisture[a]	CV	0.91	1.48		–0.01
	female Frantoio	0.92		1.45	–0.03
	female Picual	0.96		1.21	0.17
oleic acid[b]	CV	0.83	5.28		0.09
	female Frantoio	0.77		4.99	–1.17
	female Picual	0.85		4.79	–0.39
linoleic acid[b]	CV	0.85	3.53		–0.06
	female Frantoio	0.86		2.97	0.18
	female Picual	0.88		2.95	–0.02

[a] % w/w. [b] % of total fatty acids.

1490 nm arising from water absorption due to the high water content of the samples. A weaker broad absorbance centered around 1220 nm arises from second overtones of C−H and −CH=CH− stretching vibrations (*19*) probably from oil. Given that these spectra relate to frozen olives, the exact position of maxima from the major constituents present is difficult to determine. After transformation, the second derivative spectra demonstrate these absorptions more clearly with defined minima at 1025, 1215, and 1465 nm. No differences between fruits from the various female parents can be observed in the original or in the second derivative spectra. The log(1/*R*) values for each year seemed to be affected by physical sample effects rather than chemical constituents, although the second derivative plots reveal different intensities and wavelength shifts in some absorption bands between mean spectra from 1996 and 1997.

Effect of the Female Parent. The spectral data of 287 samples analyzed for fatty acids in 1997 were subjected to PCA analysis. The two first principal components account for 55 and 25%, respectively, of the variance in this spectral data set. The biplot of the PC1 and PC2 confirms that no special grouping can be observed which relates to the different female parents and data are spread uniformly along the first two PCs in all cases (**Figure 2**).

Calibration models were developed for moisture, oil, and oleic and linoleic acids using olive samples from seedling plants coming from the Arbequina parent collected in 1997. The models were first evaluated by cross-validation and then independently validated using samples collected in the same year from Frantoio and Picual parents. The correlation coefficients, bias, and predictive errors obtained for the different validation sets are shown in **Table 2**. No clear differences were observed between the results obtained by cross-validation or external validation with the other groups (female parents). Similar results were obtained when samples from Frantoio or Picual females were used to develop calibration models.

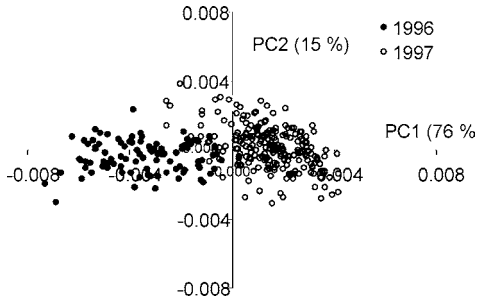

Figure 3. Biplot of scores on principal components 1 and 2 of samples analyzed for moisture and oil content by year.

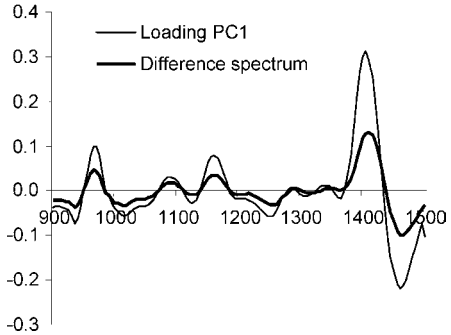

Figure 4. First loading of the PCA model developed using samples analyzed for moisture and oil content and the difference spectrum between the average second derivative of samples collected in 1996 and 1997.

These results contrast with some previously reported for other fruit species in which calibrations derived for specific cultivars validated poorly against others. The use of individual calibration models for each cultivar has been suggested for soluble solids content in apple, although acceptable accuracy was achieved by pooling all cultivar data (*20*). Similar results have also been reported for the determination of soluble solids content in peach (*21*) or melon (*22*). In red wine grapes, single variety calibrations showed a significantly improved SECV over that produced by a calibration including several varieties (*23*). In rapeseed breeding programs, the application of the calibration equations for oleic and erucic acid to the evaluation of two populations not represented in the calibration sets revealed a close relationship between NIRS and reference values. However, NIRS predictions were characterized by a high bias, which led to an underestimation of oleic acid and overestimation of erucic acid content (*24*).

Effect of the Harvest Year. To test calibration accuracy across harvest years, calibration models were developed separately with samples collected in 1996 and 1997 including all three parents. The combined models for both years involved a total of 320 samples for oil and moisture content and 434 samples for oleic and linoleic acid contents.

The score plot of the PCA model developed on spectra from samples analyzed for oil and moisture contents in the combined set ($n = 320$) is shown in **Figure 3**. A strong suggestion of sample clustering on the basis of harvest year can be seen in this figure with most of the separation being effected by the first PC, which accounts for 76% of total variation in the spectral data set. This first PC clearly shows the same pattern as the corresponding difference between the average second derivative spectrum in samples collected in 1996 and 1997 (**Figure 4**). We infer that the sample separation seen in the PC scores plot

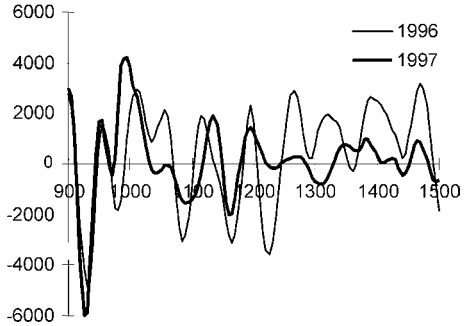

Figure 5. Regression coefficients of PLS models developed for oil content prediction using samples collected in 1996 and 1997.

arises from differences in the mean spectra and therefore mean composition in each of the two years.

PLS models developed using samples harvested in 1996 or 1997 showed different characteristics. The regression coefficients of PLS models developed for oil content prediction using samples collected in both years are shown in **Figure 5**. The differences observed between these models can be attributed to the different characteristics of the fruits collected in both years. The average proportion of oleic acid was higher in 1996 than in 1997, and the opposite trend was observed for the proportion of linoleic acid (**Table 1**). Panford and de Man (*25*) found that different wavelengths (close or similar but not exactly the same) were selected for the determination of oil content in different seed types. These variations were attributed to several factors including the different fatty acid composition of the seeds. Recently, Sato (*26*) has proposed a new estimation method for fatty acid composition based on the shift of absorption bands according to the unsaturation and length of fatty acids. Not only the chemical composition of fruits but also other characteristics such as cell size, number of cells, and amount of intercellular spaces can differ between seasons and change the physical properties of the fruits, influencing the calibration models developed with samples collected in different years (*20*, *27*). Sample temperature can also influence the absorbance of the different components, and these spectral modifications affect the prediction of the constituents in terms of higher bias. To prevent big biases, the temperature of samples should be kept constant or a calibration equation with temperature compensation should be developed (*28*, *29*).

Calibration models developed using samples harvested in 1996 or 1997 produced different results (**Table 3**). Each calibration group predicted its own validation set (cross-validation) successfully, but the errors (RMSEP and bias) increased when they were applied to another group (external validation). Only calibrations developed using samples analyzed for moisture and oil content collected in 1996 produced similar results for cross-validation and external validation. The decrease in performance of a calibration when applied to another year has been previously reported for other fruit species. It has been recommended to include as much variability in terms of orchard and season as possible for prediction of soluble solids contents in apple (*20*, *30*). In pineapple, calibration equations to predict soluble solids contents were not transferable between summer and winter fruit populations, although a calibration derived from the combined population was sufficiently robust to allow grading of fruits into two grades of sweetness (*22*). Global calibrations were successful in predicting the soluble solids contents of peaches from individual validation data sets (*21*). In mandarin, it was sufficient to add data of ca. 15 fruit to a calibration to

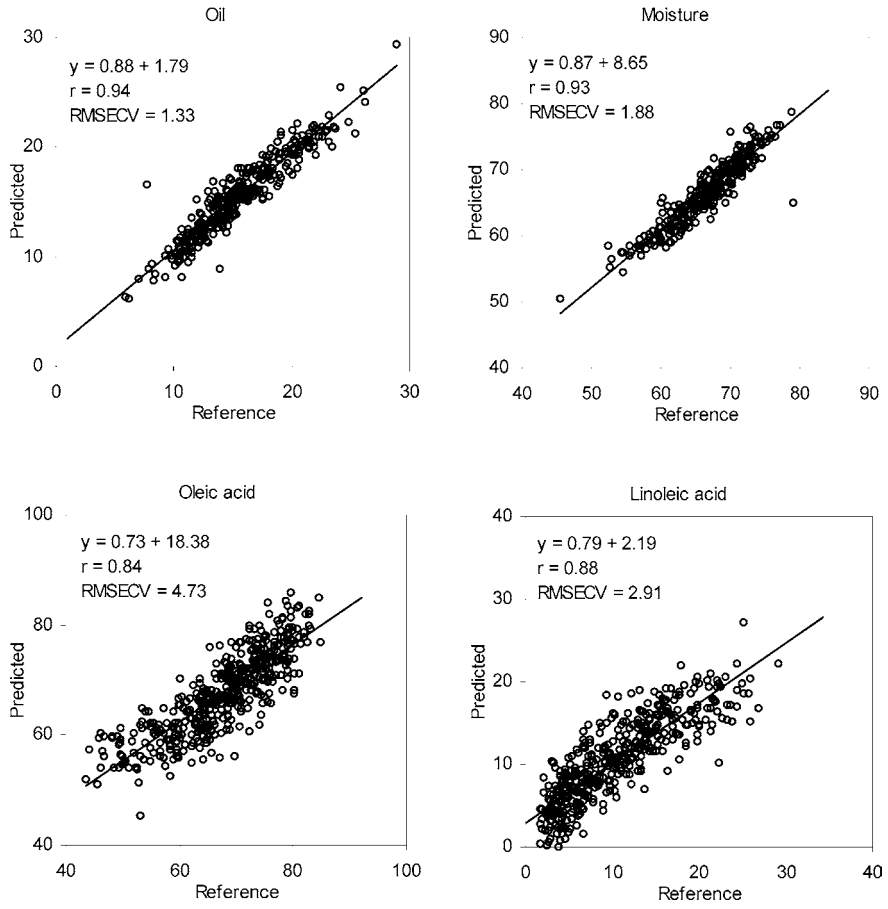

Figure 6. Predicted versus actual oil, moisture, oleic acid, and linoleic acid contents for calibration models using samples from both years (1996 + 1997).

Table 3. Cross-Validation (CV) and External Validation Results for Calibration Models by Year

constituent	calibration group	validation group	*r*	RMSECV	RMSEP	bias
oil[a]	1996	CV	0.87	1.84		–0.01
	1996	1997	0.93		1.57	0.64
	1997	CV	0.96	0.84		0.01
	1997	1996	0.82		2.41	–0.91
moisture[a]	1996	CV	0.83	2.60		0.02
	1996	1997	0.92		2.32	–0.62
	1997	CV	0.93	1.36		–0.01
	1997	1996	0.62		3.81	–0.82
oleic acid[b]	1996	CV	0.77	4.36		0.01
	1996	1997	0.73		9.62	7.31
	1997	CV	0.84	4.94		0.01
	1997	1996	0.71		6.40	2.75
linoleic acid[b]	1996	CV	0.85	2.42		0.01
	1996	1997	0.76		5.19	–2.96
	1997	CV	0.87	3.20		–0.02
	1997	1996	0.77		4.83	–2.97

[a] % w/w. [b] % of total fatty acids.

update it for use across growing season to predict soluble solids contents, improving the results in terms of SEP and bias (*27*).

In the current study, a combined calibration model (developed using 1996 and 1997 samples) predicted all constituents with *r* values ranging from 0.84 to 0.94 and SECV from 1.30 to 4.73 (**Figure 6**). Considering the compositional range of these constituents, these results produce RER values of 17.6, 18.3, 8.5, and 8.5 for oil, moisture, and oleic and linoleic acid contents, respectively. Values of *r* higher than 0.7 indicate a good fit of the predictive model, and an RER value of at least 10 has been suggested as acceptable for use in certain applications (*15*, *16*). Therefore, the models for prediction of oil and moisture content may be considered as high utility while those for oleic and linoleic acids are of moderate accuracy. The results of the combined calibration allow ranking of large numbers of seedlings on the basis of oil and oleic acid contents, as has been suggested in other tree breeding programs (*31*). This is useful because it permits selection of the most interesting genotypes for use either as a new cultivar or as breeding stock for future generations. Of the 64 samples in the combined set representing the 20% of samples with highest oil content as measured by the reference method, 58 (91%) were also ranked among the 64 samples with highest oil content by NIRS calibrations. These included all of the top 16 (5%) samples with highest oil content. A moderate accuracy was obtained for oleic acid rankings: of the 88 samples representing the 20% of samples with highest oleic acid content measured by the reference method, 56 (64%) were also ranked in the 88 samples with highest oil content predicted by NIRS, including 18 of the top 22 (82%). It seems, therefore, that NIRS could be confidently used to select the most interesting genotypes in first-stage selection on whole progeny populations where savings in costs and time could be more important than a very high accuracy.

In summary, the results obtained indicate that the performance of calibrations across new populations of samples collected from seedling plants produced by different female parents was sufficiently accurate to estimate oil, moisture, and oleic and linoleic acid contents in frozen intact olive fruits in a breeding context. Calibration models were not transferable between two years, although a calibration equation of sufficient accuracy was obtained using combined data from both years. These results confirm the usefulness of near-infrared spectroscopy analysis of intact olive fruit as a selection tool in olive breeding programs.

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Received for review February 25, 2004. Revised manuscript received May 27, 2004. Accepted June 6, 2004. The first author was supported by a fellowship from the Dirección General de Investigación y Formación Agraria y Pesquera de la Consejería de Agricultura y Pesca de la Junta de Andalucía.

JF0496853