

## Rapid Screening of Fatty Acid Alkyl Esters in Olive Oils by Time Domain Reflectometry

Annachiara Berardinelli,<sup>†</sup> Luigi Ragni,<sup>†,‡</sup> Alessandra Bendini,<sup>\*,†,‡</sup> Enrico Valli,<sup>†</sup> Lanfranco Conte,<sup>§</sup> Adriano Guarnieri,<sup>‡</sup> and Tullia Gallina Toschi<sup>†,‡</sup>

<sup>†</sup>Inter-Departmental Centre for Agri-Food Industrial Research and <sup>‡</sup>Department of Agricultural and Food Sciences (DISTAL), Alma Mater Studiorum University of Bologna, Cesena (FC), Italy

<sup>§</sup>Department di of Food Science, University of Udine, Udine, Italy

**ABSTRACT:** The main aim of the present research is to assess the possibility of quickly screening fatty acid alkyl esters (FAAE) in olive oils using time domain reflectometry (TDR) and partial least-squares (PLS) multivariate statistical analysis. Eighteen virgin olive oil samples with fatty acid alkyl ester contents and fatty acid ethyl ester/methyl ester ratios (FAEE/FAME) ranging from 3 to 100 mg kg<sup>-1</sup> and from 0.3 to 2.6, respectively, were submitted to tests with time domain resolution of 1 ps. The results obtained in test set validation demonstrated that this new and fast analytical approach is able to predict FAME, FAEE, and FAME + FAEE contents with R<sup>2</sup> values of 0.905, 0.923, and 0.927, respectively. Further measurements on mixtures between olive oil and FAAE standards confirmed that the prediction is based on a direct influence of fatty acid alkyl esters on the TDR signal. The suggested technique appeared potentially suitable for monitoring one of the most important quality attribute of the olive oil in the extraction process.

**KEYWORDS:** *fatty acid alkyl esters, food quality, olive oil, PLS, rapid screening, TDR*

### ■ INTRODUCTION

The high quality of extra virgin olive oils (EVOOs) in terms of nutritional and sensory properties is related to fruit characteristics and postharvest, manufacturing, and oil storage processes.<sup>1,2</sup> A very recent and important quality parameter for EVOOs is the content of fatty acid alkyl esters (FAAE).<sup>3</sup> Fatty acid methyl esters (FAME) and fatty acid ethyl esters (FAEE) are formed by esterification of free fatty acids (FFA) with methanol or ethanol; the reaction is favored by both temperature and concentration of reagents.<sup>4</sup> The presence of FFA depends on enzymatic activity (lipase), whereas alcohols such as methanol and ethanol are produced during alteration of olive fruits as a result of pectin degradation and sugar fermentation, respectively.<sup>5</sup> The presence of FAME and FAEE in oil increases with the duration of fruit storage time<sup>6</sup> and decreasing fruit quality.<sup>7</sup> Degraded olives, due to over-ripening or mechanical damage during storage and transport, show high contents of these components.<sup>8</sup> Recent works have shown a correlation between high FAAE values in olive oils and the main sensory defects linked to the quality of olives such as fusty-muddy, winey, and musty.<sup>9,10</sup> The analytical assessment of FAAE was proposed as a method to detect the presence of mildly deodorized olive oils in commercialized EVOOs.<sup>4,11</sup> Because mild deodorization, which is an illegal practice, neutralizes the sensory defects without producing clear chemical markers, FAAE assessment is recognized as a useful method to indirectly detect such fraud.<sup>3</sup> The content of FAAE in EVOOs is now regulated,<sup>3</sup> and the legal limit is established as ≤75 mg kg<sup>-1</sup> (sum of FAME and FAEE) or between 75 and 150 mg kg<sup>-1</sup> if the FAEE/FAME ratio is <1.5. Recent Italian legislation<sup>12</sup> also reports that, in EVOO labeled 100% Italian, the content of FAAE should not be >30 mg kg<sup>-1</sup>. Actually, alkyl ester evaluation is carried out by a time-consuming method that

involves the separation of alkyl esters by a LC silica column and the use of rather large solvent volumes, even if a new procedure, using only 3 g of silica and consequently reducing the volumes of solvents, has been recently adopted as a provisional method by the IOC. After preparative separation, capillary gas chromatography with a cold on-column injection port is used to separate FAAE with flame ionization detection for quantification. This analytical method is able to determine, at the same time, FAAE and waxes; these latter are useful as markers for the presence of pomace oil in virgin olive oil. Given the commercial importance of assessment of FAAE, a rapid method able to screen their amount could be of interest to both companies and control laboratories. In addition to official methods, the determination of authenticity and quality of EVOO can also be assessed with several spectroscopic techniques. Near-infrared,<sup>14</sup> infrared,<sup>15</sup> Raman,<sup>16</sup> and NMR<sup>17,18</sup> spectroscopy combined with multivariate analysis are the most widely used tools for the detection of adulteration in olive oils and discrimination based on quality characteristics as well as botanical and geographical origins.<sup>19,20</sup> Other rapid approaches are proposed by assessing the sensorial properties of EVOOs by electronic nose and tongue equipments.<sup>21–23</sup> Recently, FT-IR coupled with partial least-squares (PLS) methodology was developed as a useful tool for screening procedures for analytical predictions of the content of FAAE.<sup>24</sup>

Dielectric spectroscopy has been widely studied for the assessment of the dielectric properties of a variety of food products.<sup>25</sup> With regard to edible vegetable oils, experiments

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have been conducted to investigate the relationship between dielectric properties and fatty acid composition in the frequency range of 100 Hz–1 MHz<sup>26</sup> and to identify adulteration in EVOO.<sup>27</sup> Time domain reflectometry (TDR) is one of the techniques used to measure the dielectric properties of different types of liquids.<sup>28</sup> TDR is based on the analysis in the time domain of the reflection generated by a generic load as a result of the application of a fast rise time step (from ps to  $\mu$ s). The TDR technique was recently used to estimate the moisture content in granular materials (corn, soybean, bran, and coffee)<sup>29</sup> and the water content in EVOO.<sup>30</sup> Classifications of different vegetable oils were also conducted on the basis of their different dielectric characteristics. Measurement with TDR of the different dielectric behavior related to differences in chemical composition of the tested oils was proposed as an antifraud control.<sup>31</sup> Dielectric properties of vegetable oils and derived alkyl esters have been studied in depth, especially in nonfood sectors such as biofuels<sup>32</sup> and as insulation systems in power network equipments.<sup>33,34</sup> Natural esters derived from vegetable oils have been studied for their use in diesel fuels and in fuel blends<sup>35</sup> as well as in dielectric fluids with a favorable pour point.<sup>36</sup> Differences in terms of dielectric behavior (dielectric loss and permittivity) between alkyl esters derived from different oils and characterized by differences in chemical structure have been previously demonstrated.<sup>37</sup>

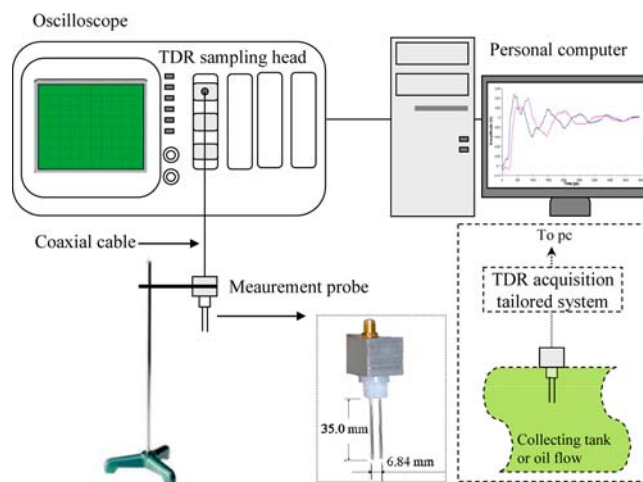
The present work aims to predict the content of FAAE in a set of EVOO samples by analyzing TDR signals with multivariate statistical tools. In a “traditional” TDR approach, the dielectric constant of the sample is calculated by analyzing the voltage reflection coefficient waveform and obtaining the travel time of the reflection. In a multivariate approach, however, all points characterizing the TDR signal (amplitude, V) can be modeled, thus overcoming the limits<sup>30</sup> shown by a “classical” approach such as the determination of the time of the reflection by geometrical methods. In the present research, principal component analysis (PCA) and PLS regressions were performed to identify the TDR regions that best describe variances in olive oil samples and to estimate the content of FAAE.

## MATERIALS AND METHODS

**Chemical Characterization of the Oil Samples.** Eighteen EVOO samples originating from Italy, Spain, and Greece, produced in 2011, were collected. The determination of FAME, FAEE, and waxes was carried out according to the official method.<sup>3,13</sup>

**TDR Instrumental Chain.** Figure 1 shows the layout of the instrumental chain used to acquire TDR signals. It was characterized by a sampling head (Tektronix, SD-24) with TDR function implemented in an oscilloscope characterized by a maximum resolution of 0.01 ps and 5120 stored data (Tektronix, 11801B) and connected to a two-terminal probe with a coaxial cable (50  $\Omega$ ; 18 GHz). The probe was made of silver-plated copper wire covered by glass pipes with the function of preventing the negative effects of electrostatic discharges. The signal capture and storage were conducted by interfacing the oscilloscope to a personal computer and using a virtual instrument developed in Labview 8.2 platform (National Instruments, Austin, TX, USA). The probe can be used for off or on-line measurement inside a tank or along the oil flow.

**TDR Measurements.** To allow signal stabilization, all acquisitions were carried out 3 h after the instruments were switched on. During measurements, conducted with an acquisition time step of 1 ps and a total acquisition time of 5120 ps, the probe was inserted in a 3 mL translucent disposable PE cuvette (Sigma, Milan, Italy) filled with 2.6 mL of EVOO. For each EVOO sample, three subsequent acquisitions (spaced by about 1 min) were carried out with the probe immersed in



**Figure 1.** TDR instrumental setup. In the inset: potential application in the oil extraction process chain.

the oil and one with the probe immersed in air (acquired as close as possible to the oil measurements). Before statistical analysis, each oil waveform was previously subtracted from the waveform acquired in air. This pretreatment was considered as a background chosen to avoid the influence of possible fluctuations of the electronic signal occurring in high-frequency instrumentation. In addition to the waveform in the time domain (amplitude, V), fast Fourier transform (FFT) frequency analysis of signals was performed to characterize the TDR signal in the frequency domain.

**Multivariate TDR Signal Analysis.** Multivariate data analysis of TDR signals (from the interface air–oil to the probe end) was conducted with The Unscrambler software tools (version 9.7, CAMO, Oslo, Norway). A preliminary PCA (full cross-validation) was conducted to identify, among the 1520 input data points, the temporal regions that best describe variances between samples in terms of FAAE content. The temporal ranges were selected after comparative analysis of the loading plots obtained by PCA together with the trend of the coefficient of determination  $R^2$  values obtained by simple linear regressions between each input data and the reference parameter FAME + FAEE ( $\text{mg kg}^{-1}$ ). PLS regression models were built for the estimation of FAME ( $\text{mg kg}^{-1}$ ), FAEE ( $\text{mg kg}^{-1}$ ), and FAME + FAEE ( $\text{mg kg}^{-1}$ ). All regressions were performed using the optimal TDR range identified after the comparison between PCA loadings as  $X$ -variables and  $R^2$  values from simple linear regressions. The data set was characterized by 54 cases (18 EVOO samples  $\times$  3 acquisitions). Full cross and test set validations were performed to estimate the accuracy of regression models. For test set validation, the 20% of the data set was randomly selected and used to validate the model built with the remaining 80% of the data set.

**Verification of the Influence of the FAEE Content on the TDR Signal.** To verify the direct influence of FAEE content on the time domain signal, acquisitions were conducted by considering one EVOO sample characterized by a low content of FAAE (lower than LOQ value) and four different mixtures of this oil prepared with methyl oleate and ethyl oleate standards (Sigma-Aldrich, Germany). The added standard concentrations were 1000 and 100  $\text{mg kg}^{-1}$  but with two different ratios, 3:1 and 1:3, of methyl oleate and ethyl oleate, respectively. These concentrations were chosen to identify the effects related to FAAE content (in terms of FAME + FAEE) and to the different concentrations of FAME and FAEE in the waveforms. Five replications were conducted to assess the reproducibility of TDR signals.

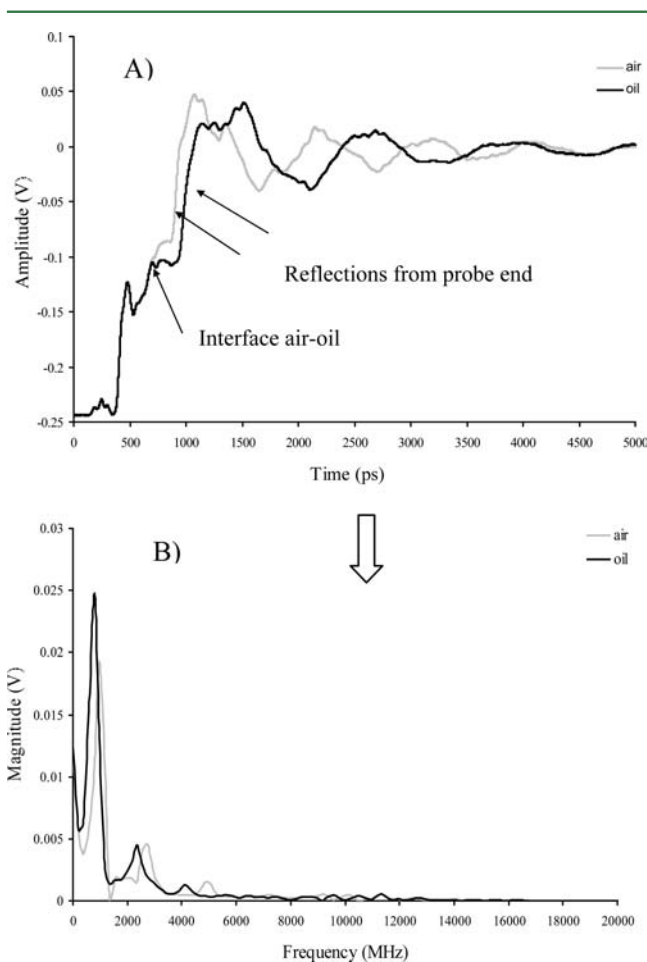
## RESULTS AND DISCUSSION

**Chemical Characterization of EVOO Samples.** The sum of FAME and FAEE ( $\text{mg kg}^{-1}$ ) and the ratio of FAEE and FAME of the 18 EVOO samples are shown in Table 1. These

**Table 1. Fatty Acid Alkyl Esters Quantified by the Official Procedure**

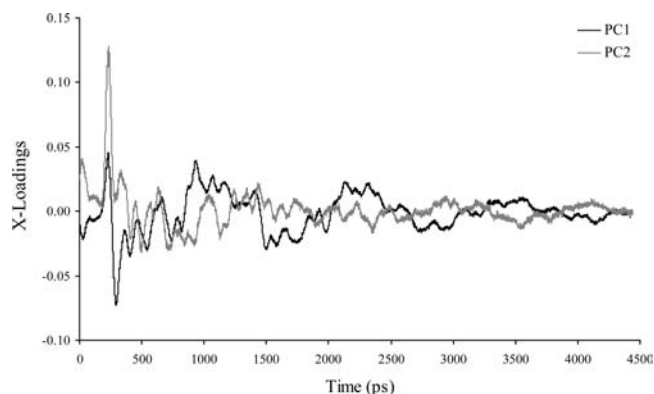
EVOO sample	FAME <sup>a</sup> (mg kg <sup>-1</sup> )	FAEE <sup>a</sup> (mg kg <sup>-1</sup> )	FAME + FAEE (mg kg <sup>-1</sup> )	FAEE/FAME
1	8.2 (0.7)	10.3 (0.8)	18.5	1.3
2	15.0 (0.7)	15.5 (0.9)	30.5	1.0
3	7.8 (0.7)	5.7 (0.8)	13.5	0.7
4	15.1 (0.7)	33.9 (1.2)	49.0	2.2
5	4.8 (0.6)	3.2 (0.7)	8.0	0.7
6	16.3 (0.9)	4.4 (0.7)	20.7	0.3
7	22.2 (1.4)	28.7 (1.1)	50.9	1.3
8	28.2 (1.9)	72.2 (1.7)	100.4	2.6
9	7.6 (0.7)	5.3 (0.7)	12.9	0.7
10	4.7 (0.6)	2.8 (0.7)	7.5	0.6
11	22.6 (1.5)	53.5 (1.4)	76.1	2.4
12	25.7 (1.7)	57.4 (1.5)	83.1	2.2
13	19.2 (1.2)	13.0 (0.9)	32.2	0.7
14	12.6 (0.5)	11.8 (0.9)	24.4	0.9
15	2.4 (0.4)	0.7 (0.7)	3.1	0.3
16	7.5 (0.7)	10.5 (0.8)	18.0	1.4
17	20.3 (1.3)	25.0 (1.0)	45.3	1.2
18	12 (0.4)	17.8 (0.9)	29.8	1.5

<sup>a</sup>Values in parentheses are standard deviations of repeatability.

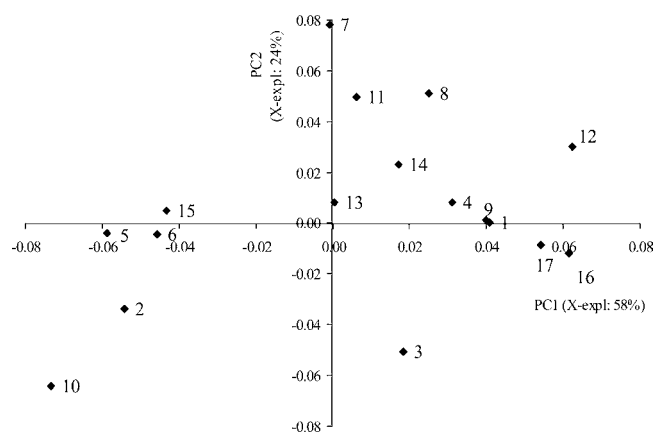


**Figure 2.** (A) Amplitude (V) of acquisitions in air and oil (EVOO sample 1) and (B) its fast Fourier transform (FFT) analysis.

values ranged from 3.1 to 100.4 mg kg<sup>-1</sup> and from 0.3 to 2.6, respectively. For 15 samples, ranges were below the legal limits for the EVOO category, whereas higher values were found for



**Figure 3.** X-Loadings for principal components PC1 ( $x\text{-exp} = 58\%$ ) and PC2 ( $x\text{-exp} = 24\%$ ).

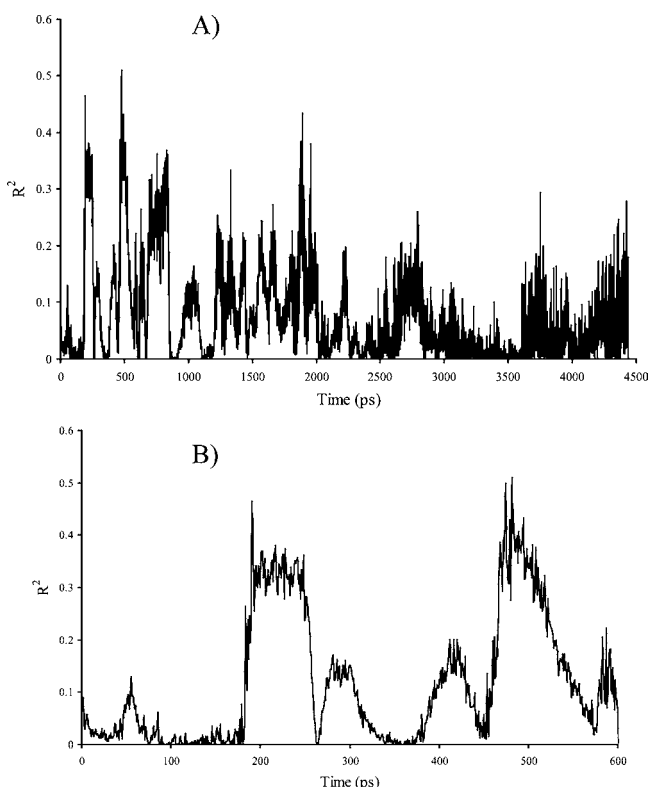


**Figure 4.** Scores plot of the EVOO samples for the first two principal components (mean values of three replications, see Table 1).

three samples (8, 11, and 12). With regard to the content of waxes (C40–C46), the values ranged from 35 to 142 mg kg<sup>-1</sup>, and thus all samples were well below the legal limit of 250 mg kg<sup>-1</sup>.<sup>3</sup>

**TDR Signals.** An example of acquired waveforms (amplitude, V) in the time domain for the measurements conducted in oil and air is shown in Figure 2A (EVOO sample 1, see Table 1). The different behavior observed between the reflections from the probe end in oil and air was due to the different dielectric properties of the air and oil. In a traditional TDR approach, the first rise step after the interface point is used to calculate the travel time of the reflection and the dielectric constant  $k$  of the analyzed system.<sup>38</sup> This traditional TDR approach can be unsuccessful when dielectric differences are not large and not graphically evident, so that the error due to the estimation of dielectric parameters is relevant.<sup>30</sup> The relative FFT is shown in Figure 2B. The frequency analysis revealed that TDR signals are characterized by dominant peaks at 781 (oil) and 976 (air) MHz. Other components can be observed at 2343 and 4101 MHz for TDR oil signals and at 2734 and 4882 MHz for TDR air signals. The first point of each waveform, obtained by subtracting the signal acquired in air to the signal in oil, corresponds to the interface air–oil that can be located after about 680 ps. This point was arbitrarily identified where the ratio between the two amplitudes (V) (in air and in oil) was  $>0.005$ .

**Multivariate Data Analysis: Principal Component Analysis.** The results of PCA conducted on TDR signals are



**Figure 5.** Values of the coefficient of determination  $R^2$  for simple linear regressions obtained for FAME + FAEE for all data points (A) and for data related to the first 600 ps (B).

summarized in Figures 3 and 4 in terms of  $X$ -loadings and scores plot, respectively, of the first two principal components (full cross-validation). To visually understand the results of the modeling, the scores plot shows the mean values of the three replications conducted on the same EVOO sample.

As explained above, the first part of the signal corresponding to the antecedent portion of the interface air–oil (up to 680 ps) (see Figure 2A) was not considered in the analysis. For both PC1 and PC2 components, the highest  $X$ -loading values were observed in the signal region corresponding to the first rise step after the interface air–oil (up to 600 ps after the interface point, see Figure 2A).

In the relative scores plot given in Figure 4, the first component appeared to roughly model the samples according to the FFAE content (in terms of FAME + FAEE,  $\text{mg kg}^{-1}$ ). The second component appeared to separate the samples characterized by the highest values of FFAE (in terms of FAME + FAEE,  $\text{mg kg}^{-1}$ ) (7, 8, 11, 12, and 14; see Table 1) from the remaining data set.

In the present research, the results of the scores plot were used only to discuss the information contained in TDR signal in relations to the FFAE content. No further improvements of

the PCA model, such as outlier identification or use of the optimal TDR signal range, were considered.

The values of the coefficient of determination  $R^2$  obtained by simple linear regressions between each input data (from the interface air–oil) and the reference FAME + FAEE ( $\text{mg kg}^{-1}$ ) are given in Figure 5. A zoom of the first 600 ps, where the  $R^2$  values are more robust, is also reported in the same figure (Figure 5B). As discussed for  $X$ -loadings, the highest values of  $R^2$  can be observed in the first rise step region, and particularly up to about 600 ps from the oil–air interface (see Figure 2A). According to these considerations, for PLS regression models the portion of the TDR signals starting from the air–oil interface and ending immediately after the rise step (after about 600 ps from the interface) was considered.

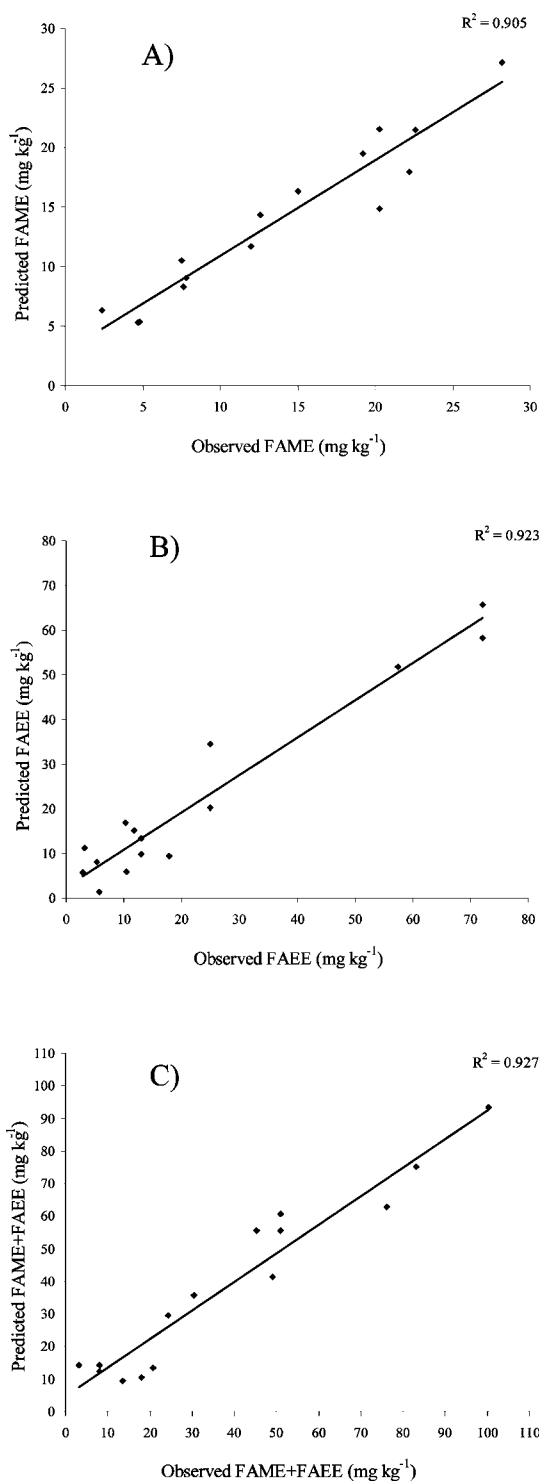
**Multivariate Data Analysis: Partial Least-Squares Regressions.** The results of the PLS regression analysis are summarized in Table 2 for the dependent variables FAME, FAEE, and FAME + FAEE ( $\text{mg kg}^{-1}$ ). In test set validation,  $R^2$  values of 0.905 (RMSE = 2.4  $\text{mg kg}^{-1}$ ), 0.923 (RMSE = 6.5  $\text{mg kg}^{-1}$ ), and 0.927 (RMSE = 7.9  $\text{mg kg}^{-1}$ ) were, respectively, obtained for the prediction of FAME, FAEE, and FAME + FAEE error of (Figure 6). Predicted versus observed values for FAME ( $\text{mg kg}^{-1}$ ), FAEE ( $\text{mg kg}^{-1}$ ), and FAME + FAEE ( $\text{mg kg}^{-1}$ ) are, respectively, shown in panels A, B, and C of Figure 6 (test set validation). In terms of accuracy and FAME + FAEE content, similar results were obtained by applying the proposed rapid method to another set of 10 different EVOOs ranging from 7 to 38  $\text{mg kg}^{-1}$ . In full cross-validation,  $R^2$  values of 0.917 were observed with a root-mean-square error of 2.7  $\text{mg kg}^{-1}$  (data not shown). An attempt to predict the wax amount in EVOO by TDR (not discussed in this study) was also made, but failed. The coefficient of determination of the PLS models was 0.891 and 0.585 (cross and test validations); PCA did not show a clear separation between samples. The result was not surprising considering that the dielectric constant of wax is very close to those of other fatty acids and that the variability in wax content in the data set was low.

**Verification of the Influence of FFAE Content on the TDR Signal.** The TDR signals acquired on the different EVOO mixtures over 600 ps are shown in Figure 7 (from the air–oil interface to the first rise step). In particular, the waveforms are the result of subtraction from the signal acquired in air of the signal acquired in oil. The detail shown in Figure 7 corresponds to the first portion of the first rise step after the air oil interface, which appeared in the previous sections as the most correlated region with FFAE content. Although referred to small voltage differences (i.e., a few mV), a clearly different dielectric behavior according to the different content of FFAE (both the sum and the single FAME and FAEE) can be observed. As is evident from Figure 7, the spectral differences were very small. Thus, the suggested technique requires conditions of high stability for measurement. In this sense, the temperature and

**Table 2.** PLS Model Accuracy for the Prediction of FAME, FAEE, and FAME + FAEE

parameter	calibration			full cross-validation			test set validation		
	$R^2$	LV <sup>a</sup>	RMSE <sup>b</sup>	$R^2$	LV <sup>a</sup>	RMSE <sup>b</sup>	$R^2$	LV <sup>a</sup>	RMSE <sup>b</sup>
FAME ( $\text{mg kg}^{-1}$ )	0.996	8	0.4	0.909	8	2.3	0.905	8	2.4
FAEE ( $\text{mg kg}^{-1}$ )	0.996	8	1.3	0.928	8	5.7	0.923	8	6.5
FAME + FAEE ( $\text{mg kg}^{-1}$ )	0.994	8	2.1	0.934	8	7.1	0.927	8	7.9

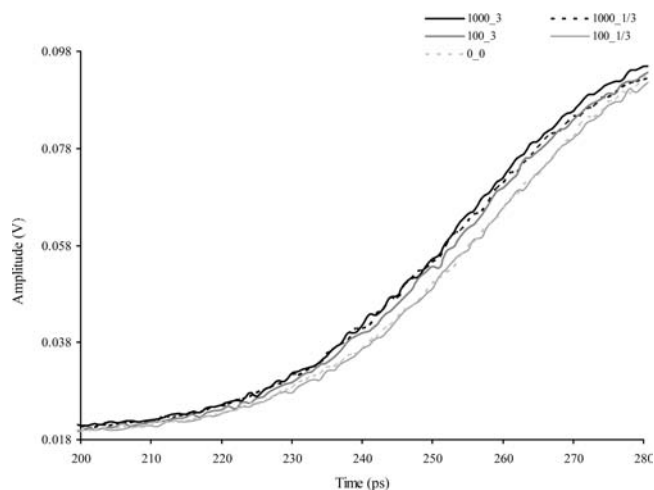
<sup>a</sup>LV, linear vectors. <sup>b</sup>RMSE, root-mean-square error ( $\text{mg kg}^{-1}$ ).



**Figure 6.** Predicted versus observed values in test set validation (see Table 2) for (A) FAME (mg kg<sup>-1</sup>), (B) FAEE (mg kg<sup>-1</sup>), and (C) FAME + FAEE (mg kg<sup>-1</sup>).

level of oil, electrical stability, and warming of instrumentation can play crucial roles.

It is possible to summarize the main results obtained herein as follows: (i) Time domain reflectometry, supported by PLS statistical analysis, appears to be a promising technique for assessment of FAAE in olive oil samples. (ii) The predictive power of the content of FAME, FAEE, and FAME + FAEE is characterized by  $R^2$  values of 0.905, 0.923, and 0.927 and RMSE



**Figure 7.** Detail of the waveforms obtained by subtraction of oil from the air acquisitions (0, 100, and 1000 mg kg<sup>-1</sup> of alkylesters with two different ratios, 3:1 and 1:3, of methyl oleate and ethyl oleate).

of the order of 2.4, 6.5, and 7.9 mg kg<sup>-1</sup> in test set validation, respectively. (iii) TDR measurements carried out in EVOO by adding FAAE demonstrated that they have a direct influence on the electrical signal. (iv) The observed differences in the signal response are minimal, so that high resolution and stability of the instrumental chain become essential for good prediction. (v) At the moment, TDR can be considered as a promising tool for rapid and environmentally friendly screening of the content of FAAE in olive oil samples.

## AUTHOR INFORMATION

### Corresponding Author

\*(A. Bendini) Phone: +39 0547 33 8121. Fax: +39 0547 382348. E-mail: [alessandra.bendini@unibo.it](mailto:alessandra.bendini@unibo.it).

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### Notes

The authors declare no competing financial interest.

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