Determination of Olive Oil Free Fatty Acid by Fourier Transform Infrared Spectroscopy

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ABSTRACT: A new procedure for determining free fatty acids (FFA) in olive oil based on spectroscopic Fourier transform infrared–attenuated total reflectance spectroscopy measurements is proposed. The range of FFA contents of samples was extended by adding oleic acid to several virgin and pure olive oils, from 0.1 to 2.1%. Calibration models were constructed using partial least-squares regression (PLSR). Two wavenumber ranges (1775–1689 cm^{-1} and 1480–1050 cm^{-1}) and several pretreatments [first and second derivative; standard normal variate (SNV)] were tested. To obtain good results, splitting of the calibration range into two concentration intervals (0.1 to 0.5% and 0.5 to 2.1%) was needed. The use of SNV as a pretreatment allows one to analyze samples of different origins. The best results were those obtained in the 1775–1689 cm^{-1} range, using 3 PLSR components. In both concentration ranges, at a confidence interval of $\alpha = 0.05$, no significant differences between the reference values and the calculated values were observed. Reliability of the calibration vs. stressed oil samples was tested, obtaining satisfactory results. The developed method was rapid, with a total analysis time of 5 min; it is environment-friendly, and it is applicable to samples of different categories (extra virgin, virgin, pure, and pomace oil).

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KEY WORDS: ATR, free fatty acid, FTIR, multivariate calibration, olive oil, PLSR, quality control.

Olive oil is an essential component of the Mediterranean diet. The European Union (EU) has established different categories of olive oil according to the production process (1). In order to ensure the quality within the different categories, several analyses should be made. One of the important methods to determine olive oil quality is the free fatty acid (FFA) content.

Official wet chemistry methods are time consuming, labor intensive, and require skilled technicians and reagents that are costly to dispose of safely. This can be avoided by using a spectroscopic technique which offers the potential for rapid determination of large numbers of samples by unskilled workers with minimal use and disposal of costly solvents and chemicals. Most vegetable oil spectroscopic applications focus on detecting adulteration (2), determining classification (3), and establishing geographical origins (4). Also, a few quantitative determinations have been done without any previous separation techniques. Thus, Fourier transform infrared (FTIR) spectroscopy has become an alternative to these techniques because of its simplicity in sample handling and unnecessary pretreatment. Several lipid analysis FTIR methods (5–8) have been reported, such as the monitoring of the oxidation of edible oils, the determination of their *cis* and *trans* content, peroxide values, and free fatty acids.

The need to develop new simplified routine methods has resulted in little attention given to the selection of samples and their calibration; however, this process is essential if reliable predictions are desired. Thus, an overfitted calibration model may provide good results for the calibration set but poor predictive ability for similar samples if sufficient variability is not included in the calibration process. Therefore, proper development of analytical methods in this context requires careful use of multivariate calibration methods to obtain reliable results. The application to external prediction samples and its comparison to official reference methods improves the consistency of the results (9,10).

The objective of the research was to develop a rapid FTIR method for olive oil FFA analysis using multivariate treatments of the data obtained by direct attenuated total reflectance (ATR)–FTIR measurements with the aim of replacing laborious official methods when a large number of samples must be analyzed.

MATERIALS AND METHODS

Samples. In order to ensure that the study would be representative of the different types and categories of commercially available olive oil, samples of different origins and ages were analyzed. A total of 87 olive oil samples of different categories, with FFA contents between 0.1 and 2.1% were analyzed, including 36 virgin, 40 pure, and 11 pomace olive oil samples. Although the FFA contents of extra virgin olive oils range from 0 and 1%, in an effort to expand the calibration interval, several samples were prepared with added oleic acid to increase the range of FFA contents studied. Also, 13 additional samples were prepared by heating several olive oil samples at 250°C for 2–3 h to obtain thermally stressed samples.

Chemical procedures. FFA contents were determined by using the reference method (11). Briefly, this consists of a

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nonaqueous titration of the sample in a diethyl ether–ethanol medium (1:1), using an ethanolic solution of 0.1 M KOH as the standard reagent.

Physical measurements. FTIR spectra were recorded in the ATR mode on a Perkin-Elmer 16PC (Norwalk, CT) spectrophotometer equipped with a Graseby Specac p/n 11130 (Kent, United Kingdom) horizontal ATR device. This is a channeling device suitable for viscous fluids that uses a 45°ZnSe parallelogram with mirrored angle faces providing six reflections on the crystal surface. The spectrophotometer was run under software 16PC Instrument Control v. 2.20 (Perkin-Elmer); and IR Data Manager v. 3.34 (Perkin-Elmer), which was used for recording and processing the spectra.

A sample volume of 1–1.5 mL was uniformly spread throughout the crystal surface to obtain the spectra. Each spectrum was the result of 50 scans performed at 2 cm^{-1} intervals over the wavenumber range from 4000 to 650 cm^{-1}. One spectrum per sample was recorded, using air as a reference. (An example is shown in Fig. 1.) Before each spectrum was recorded, the ZnSe crystal was wiped with cellulose tissue soaked in an aqueous solution 1% of Triton X-100 and then rinsed with distilled water and isopropyl alcohol.

Data processing. In addition to absorbance data, we assayed various spectral treatments to avoid baseline shifts arising from scatter, viz. first and second derivative, and standard normal variate (SNV) (12,13).

Calibration models were constructed with partial least-squares regression (PLSR) (14,15) from autoscaled data. PLSR and first- and second-derivative treatments of the spectra were done with the aid of Unscrambler v. 6.1 (Camo ASA, Oslo, Norway).

Cross-validation process was used in model validation, with as many validation subsets as there were samples included in the calibration matrix (leave-one-out method) (16). For the determination of the optimal number of principal components, minimum value of Mean Square Error of Cross Validation, MSECV was used. For each PLSR-calculated component k, MSECV is defined in Equation 1 as:

$$\mathrm{MSECV}_k = \frac{\sum_{i=1}^{N} (y_{i\,\mathrm{calc}} - y_{i\,\mathrm{ref}})^2}{N} \quad [1]$$

where $y_{i\mathrm{calc}}$ is the concentration of sample i as calculated by the model, $y_{i\mathrm{ref}}$ the reference value, and N the total number of samples used for calibration. But this criterion occasionally results in overfitted models. To avoid this undesirable effect, we subjected the number of PLSR components required to obtain the minimal value of MSECV to a backward regression procedure over a confidence interval ($\alpha = 0.25$).

In order to determine the most suitable wavenumber range and spectral mode, we applied the different calibration models to a set of prediction samples which were not used in constructing the models. The results were compared in terms of the Root Mean Square Error RMSE (Equation 2).

$$\mathrm{RMSE} = \sqrt{\frac{\sum_{i=1}^{i} (y_{i\,\mathrm{calc}} - y_{i\,\mathrm{ref}})^2}{I}} \quad [2]$$

where $y_{i\,\mathrm{calc}}$ is the FFA content calculated by PLSR, $y_{i\,\mathrm{ref}}$ the reference value, and I the total number of samples for which RMSE was calculated.

Wavenumber selection. Two spectral ranges were studied. The first range, 1775–1689 cm^{-1}, exhibited a strong band at 1748 cm^{-1} corresponding to absorption by carbonyl bonds in acyl glycerides; another at 1710 cm^{-1}, due to the carbonyl bonds in free fatty acids, strongly overlapped with the previous one (Fig. 1). In order to complement the spectral information and assess its potential influence on quantitation, an additional range (1480–1050 cm^{-1}) was selected. It provided information about asymmetric stretching in methyl and methylene groups (~1465 cm^{-1}), a band due to stretching in the C–O bonds of aliphatic esters (~1160 cm^{-1}), and a third band corresponding to aliphatic methylene groups [C–$(CH_2)_n$–C, ~721 cm^{-1}]. All ranges were confirmed by the direct observation of the PLSR loadings obtained after the calculations.

Selection of samples. For a calibration model to be reliable it must be representative of the system and thus include its expected natural variability. In order to ensure selection of samples encompassing every possible source of variability, we grouped them according to similarities; for this, we performed a cluster analysis and chose samples from the different clusters for inclusion in the calibration set (17). Calculations were carried out using the statistical software package SPSS for Windows v. 7.5.2 (SPSS Inc., Chicago, IL), using Principal Component data.

The SNV spectra of 65 different samples of oil were subjected to Principal Component Analysis (PCA) over the wavenumber range 1480–700 cm^{-1}, which comprises the fingerprint region and is the spectral region which exhibits the

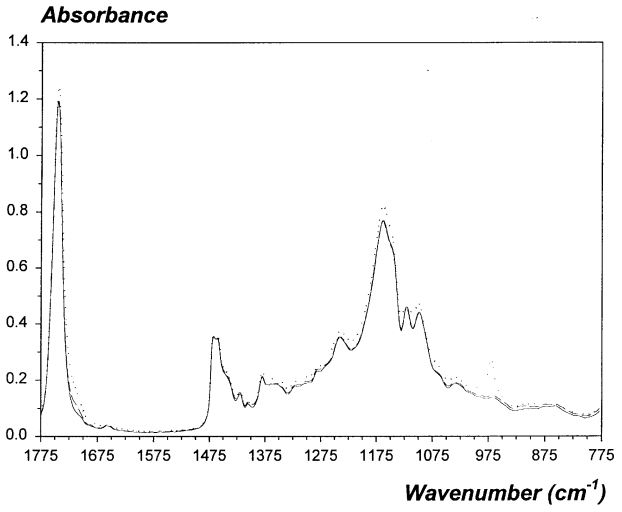

FIG. 1. Attenuated total reflectance–Fourier transform infrared absorbance spectra for virgin olive oils of different free fatty acid contents: low (solid line); high (dashed line); thermally stressed sample (dotted line).

most marked differences among samples, as can be seen in Figure 1.

RESULTS AND DISCUSSION

Once the PCA using the SNV data was done, the scores provided by the first eight principal components (PC), which accounted for 85.9% of the variance in the body of spectra, were subjected to cluster analysis using complete linkage as the agglomeration technique. As can be seen from Figure 2, samples formed seven different clusters at a distance of 11 units; the clusters reflected the different oil categories (virgin, pure, and olive pomace) and also the differences due to the individual samples (geographical procedence, noncorrected low turbidity effects, etc.), which are useful for incorporating maximal variability to the calibration. By using this information, samples of each class were selected for their inclusion in the calibration, and prediction sets in all cases were studied.

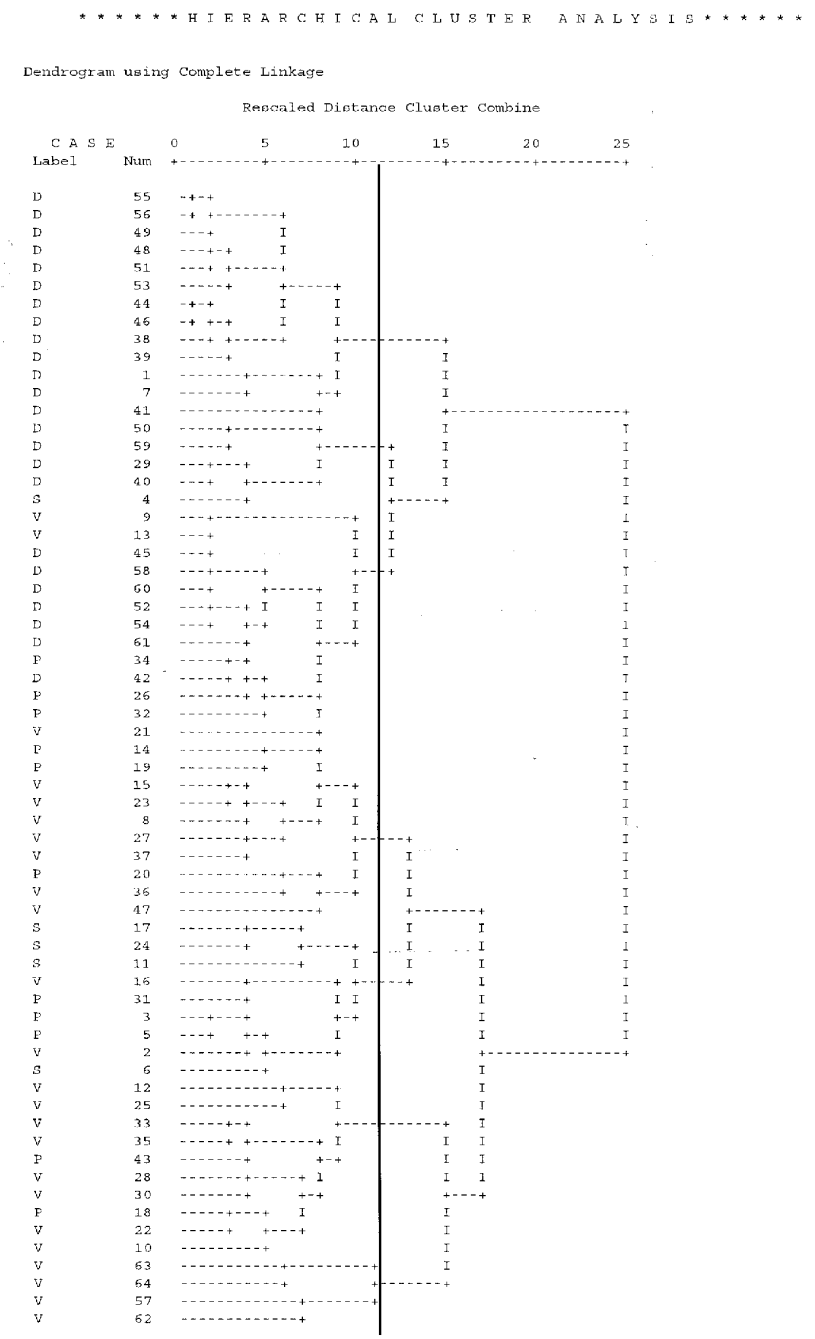

FIG. 2. Selection of samples. Dendogram obtained by using complete linkage as agglomeration technique. Samples: D (olive oils spiked with oleic acid), V (virgin olive oils), P (pure olive oils), and S (pomace olive oils).

TABLE 1
$RMSEC_1$ and $RMSEP_1$ Values Provided by the Different Calibration Models Using Different Numbers of PC[a]

Range	Spectral mode	PC	$RMSEC_1$	$RMSEP_1$
1775–1689 cm^{-1}	Absorbance	4	0.044	0.072
	First derivative	3	0.052	0.071
	Second derivative	3	0.069	0.108
	SNV	3	0.041	0.111
1775–1689 + 1480–1050 cm^{-1}	Absorbance	5	0.053	0.100
	First derivative	4	0.048	0.121
	Second derivative	5	0.044	0.181
	SNV	3	0.067	0.138

[a]$RMSEC_1$, root mean square error of calibration for set 1; $RMSEP_1$, root mean square error of prediction for set 1; PC, principal component; SNV, standard normal variate.

From the clusters of the obtained dendrogram, a calibration set C_1 consisting of 37 samples was constructed with 16 virgin oil, 17 pure, and 4 pomace olive oil samples. They uniformly spanned the FFA range from 0.1 to 2.12%. In the same way, the prediction set P_1 was selected consisting of 22 samples. This set was used to assess the predictive capacity of the different calibration models and compare the results provided by the different spectral modes and wavenumber ranges.

Table 1 gives the RMSE for sets C_1 ($RMSEC_1$) and P_1 ($RMSEP_1$) in the wavenumber ranges studied. As can be seen, prediction errors were, in general, much greater than calibration errors, thus suggesting that the models were overfitted. Inclusion of the 1480–1050 cm^{-1} range increased the error for the prediction set because absorption by other products present in the sample caused spectral changes similar to those produced by FFA. For this reason it was not recommended to include this range in this and next models.

Figure 3 shows the variation of the absolute error as a function of concentration for the calibration (C_1) and prediction set (P_1) of the model based on the wavenumber range 1775–1689 cm^{-1} in the spectral mode selected "absorbance." The two horizontal lines show the laboratory error made in applying the reference method. It was calculated from 57 titrations of 19 different samples and expressed as standard deviation according to ISO Standards (18) resulting in a value of 0.09%. As can be seen, dispersion in the results obtained by prediction using PLSR model was not constant; in fact, it was slightly higher below a FFA content of 0.5%. In order to enhance the accuracy of the method and to reduce errors in the low FFA content range, it was necessary to split the concentration range into two subranges and obtain different calibration models (19).

Model for FFA contents below 0.5%. Thirty-six samples with FFA contents below 0.5% belonging to different clusters were selected as the calibration set (C_2). The same criterion was used to select 14 samples for a prediction set (P_2).

Table 2 shows the RMSE values obtained for the calibration set ($RMSEC_2$) and prediction set ($RMSEP_2$) by using the different spectral modes in the wavenumber range 1775–1689 cm^{-1}. As can be seen, using first- and second-derivative spectra resulted in poorer predictions. Second-derivative spectra increased background noise leading to an increase of the number of PC required, which lowered predictive capacity.

The quantitative errors obtained in the 1775–1689 cm^{-1} range were similar with both absorbance and SNV-corrected spectra; however, the latter provided more simple models involving three PC only. A plot of calculated values from SNV-corrected spectra against reference values at a significance level ($\alpha = 0.05$) was a straight line of slope 0.89 ± 0.11, intercept 0.03 ± 0.03 and regression coefficient $r = 0.943$. A t-test on the differences between reference values and those calculated for the prediction set confirmed the absence of significant differences between the two ($t_{exp} = 0.39$, was smaller than $t_{critical}$ at a significance level $\alpha = 0.05$). Figure 4 shows the calculated residuals for sets C_2 and P_2; as can be seen, they were smaller than ±0.1% for all samples.

Model for FFA contents between 0.5 and 2.1%. From available samples spanning the 0.5 to 2.1% FFA content range, 25 were chosen to form calibration set C_3 according to the same criterion as in previous sections. The corresponding validation set, P_3, consisted of eight samples.

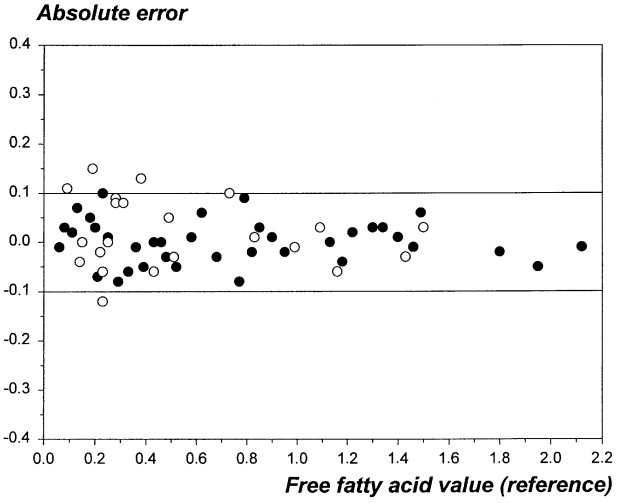

FIG. 3. Absolute errors for calibration (●) and prediction samples (○) obtained in the absorbance mode and wavenumber range 1775–1689 cm^{-1}.

TABLE 2
$RMSEC_2$ and $RMSEP_2$ Values Provided by the Different Calibration Models for the Free Fatty Acid Contents from 0.1 to 0.5% Using Different Number of PC[a]

Range	Spectral mode	PC	$RMSEC_2$	$RMSEP_2$
1775–1689 cm^{-1}	Absorbance	4	0.042	0.050
	First derivative	3	0.054	0.068
	Second derivative	4	0.053	0.115
	SNV	3	0.044	0.057

[a]For abbreviations see Table 1.

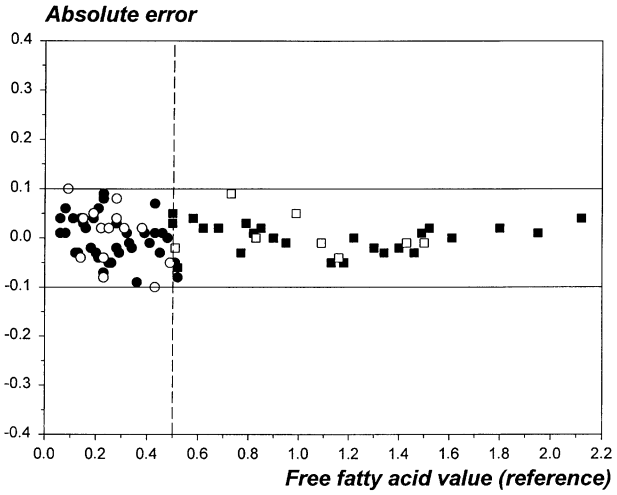

FIG. 4. Absolute errors obtained in the standard normal variate mode and wavenumber range 1775–1689 cm^{-1}. Lower free fatty acid (FFA) contents subrange: calibration, C_2 (●) and prediction, P_2 (○). Upper FFA contents subrange: calibration, C_3 (■) and prediction, P_3 (□).

Table 3 gives the $RMSEC_3$ and $RMSEP_3$ values obtained by using the different spectral modes tested over the wavelength range 1775–1689 cm^{-1}. As in the previous case, the SNV treatment provided a simpler calibration model than did absorbance data, while preserving the predictive capacity. A plot of SNV calculated values against reference values at a significance level $\alpha = 0.05$ was a straight line of slope 1.0 ± 0.03, intercept 0.00 ± 0.03 and regression coefficient r = 0.998. As in the previous case, a difference t-test on the prediction set provided a t_{exp} value (0.43) that was smaller than $t_{critical}$ at the same confidence level. Figure 4 shows the residuals for the calibration (C_3) and prediction set (P_3).

External prediction. After the optimal models for each FFA content zone were chosen, their predictive capacity was checked on 22 samples that had not yet been used (set P). Each sample was first quantified by using the model for FFA contents between 0.1 and 2.1%. This provided rough estimates of the acidities of the unknown samples, but also provided a means for classifying them with a view to a subsequent, more precise determination of their FFA by using the most suitable model in each case.

The RMSE value obtained by applying the model for the entire FFA scale to the 22 samples was 0.100 and thus much greater than that provided by the same model for sets C_1 and P_1. This further testifies to its unfitness for the purpose. Using the model for the 0.1–0.5% range to quantify the samples of FFA below 0.5% resulted in an RMSE value of 0.046, which was similar to those for sets C_2 and P_2. The samples of FFA above 0.5% quantified with the model for the 0.5–2.1% range provided an RMSE value of 0.019, similar to those for sets C_3 and P_3. Figure 5 shows the residuals for this sample set as obtained by using the entire FFA range and its subranges. The latter provided satisfactory results, with residuals less than ± 0.1%.

Predictive capacity was also checked on a set of 13 samples of thermally stressed olive oil. Figure 5 shows the errors obtained by quantifying the oxidized samples using the above-described models for the natural olive oil samples with SNV-corrected spectra. It was observed that the same calibration model can be applied to fresh and thermally stressed samples, which was not possible using absorbance models.

It has been shown that the joint use of ATR–FTIR spectrophotometry and PLSR calibration has allowed us to develop an alternative to official methods that determines FFA in olive oils of different types and origin. The use of some

TABLE 3
$RMSEC_3$ and $RMSEP_3$ Values Provided by the Different Calibration Models for the Free Fatty Acid Contents from 0.5 to 2.1% Using Different Numbers of PC[a]

Range	Spectral mode	PC	$RMSEC_3$	$RMSEP_3$
1775–1689 cm^{-1}	Absorbance	4	0.036	0.049
	First derivative	3	0.034	0.058
	Second derivative	4	0.054	0.054
	SNV	3	0.029	0.040

[a]For abbreviations see Table 1.

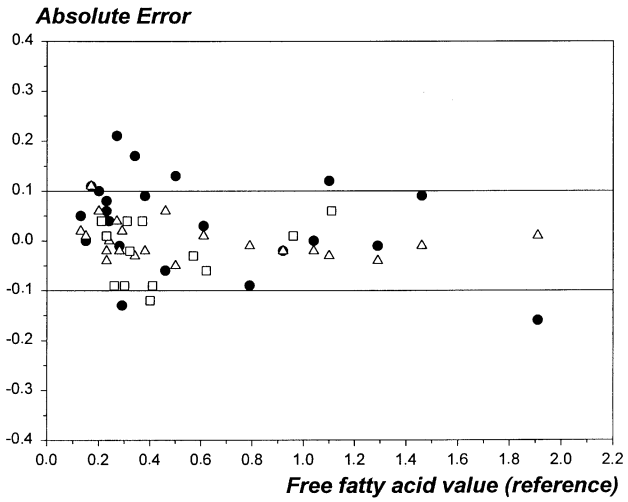

FIG. 5. Absolute errors for the prediction samples (set P) obtained by modeling the entire FFA contents range (●). Absolute errors for the prediction samples (set P) (△) and thermally stressed samples (□) using splitted range.

pretreatments, such as SNV, leads to similar results as using absorbance data with standard samples but provides less complicated models that can be directly applied to different kinds of samples, including thermally stressed oils.

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