J Am Oil Chem Soc (2009) 86:507–511
DOI 10.1007/s11746-009-1389-0

ORIGINAL PAPER

Rapid Determination of Free Fatty Acid in Extra Virgin Olive Oil by Raman Spectroscopy and Multivariate Analysis

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Received: 10 September 2008 / Revised: 9 April 2009 / Accepted: 9 April 2009 / Published online: 5 May 2009
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Abstract We introduce a visible Raman spectroscopic method for determining the free fatty acid (FFA) content of extra virgin olive oil with the aid of multivariate analysis. Oleic acid was used to increase the FFA content in extra virgin olive oil up to 0.80% in order to extend the calibration span. For calibration purposes, titration was carried out to determine the concentration of FFA for the investigated oil samples. As calibration model for the FFA content (FFA%), a partial least squares (PLS) regression was applied. The accuracy of the Raman calibration model was estimated using the root mean square error (RMSE) of calibration and validation and the correlation coefficient (R^2) between actual and predicted values. The calibration curve of actual FFA% obtained by titration versus predicted values based on Raman spectra was established for different spectral regions. The spectral window (945–1600 cm^{-1}), which includes carotenoid bands, was found to be a useful fingerprint region being statistically significant for the prediction of the FFA%. High R^2 and small RMSE values for calibration and validation could be obtained, respectively.

Keywords Raman spectroscopy · Chemometrics · Free fatty acid · Olive oil

Introduction

Olive oil is a complex compound that contains fatty acids, vitamins, pigments, volatile and anti-oxidative components [1]. The hydrolysis of oil results in the formation of free fatty acid (FFA) and glycerol residues [2]. Many factors trigger the formation of FFA in the oil raw material (e.g. olive fruits). These include, but are not limited to fruit-fly infestation of fruits, fungal diseases in the fruit, and delay periods between harvesting and extraction, especially when storage is not cautiously and properly achieved or when the fruits were damaged during harvesting [3]. FFA is one of the major causes of flavor deterioration and shelf life decrease of oil [4]. For that reason, it is one of the most fundamental parameters used to evaluate the quality of olive oil. The FFA content in the form of oleic acid is used to classify olive oil within different categories. According to the International Olive Oil Council, extra virgin olive oil is the oil extracted from olive fruit mechanically without any thermal or chemical treatment and has a maximum of 1% FFA, while olive oil with 3.3% FFA is considered unsuitable for human consumption and must be refined prior to consumption [5]. The traditional method for FFA determination based on titration is time consuming, and requires a skilled analyst. Different other methods are used to measure FFA contents, such as pH-metric, chromatographic and colorimetric techniques [6, 7], which are quite sensitive and precise. Recently, spectroscopic techniques have been recognized as versatile analytical tools in oil quality control. Fourier transform infrared absorption spectroscopy (FTIR) has been used for FFA determination of different types of oils [8–11]. In these previous works, FFA could be determined with 0.5% or under optimum conditions 0.1% sensitivity for FFA. Moreover, many of these works were invasive in the sense that the oils underwent saponification reactions before the IR detection was possible.

Raman scattering arising from vibrational transitions in molecules provides useful information about the molecular

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structure via well-defined and separated spectral fingerprints. In food analysis, Fourier transform Raman spectroscopy (FTR) has been demonstrated as a potential analytical tool in oil authentication [12–14] and classification [15], and in the detection of frying oil deterioration [16], oil oxidation [17, 18] and adulteration [19–21]. To the best of our knowledge, only one research work was reported for FFA determination ranged between 0.2 and 6.14% in olive oil and olives using FT-Raman scattering with near infrared (NIR) excitation at 1,064 nm [3]. The aim of our research work presented in the following was to evaluate the potential of Raman spectroscopy as a rapid and nondestructive tool, in combination with chemometric analysis, to quantify the FFA content in olive oil using visible excitation at 514.5 nm. This method offers many advantages over the NIR FT-Raman used in the previous studies, including high sensitivity and high spectral resolution achieved using a simple Raman setup. Moreover, the visible excitation source can help to resonantly excite (electronic resonance) vibrational bands of many other important constituents of oils such as pigment molecules, of which carotenoids are of particular interest. For instance, an intense signal due to carotenoids can be detected even at very low concentrations [22].

Materials and Methods

Raman Experiment

The samples tested were contained in 1,800-μl quartz cuvettes (Starna) and illuminated by the 514.5 nm line of an Ar-ion laser (Coherent, Inova 308 Series) with an excitation power of 10 mW on the sample. The laser was focused within the sample using an inverted microscope setup equipped with a 10× ultra long working distance objective (10× ULWD, N.A 0.20; Olympus). The scattered signal was then recorded at a 180° backscattering geometry and dispersed by a single monochromator (TRIAX 550, Jobin Yvon) using a 1,200 grooves/mm diffraction grating and an entrance slit width of 200 μm. The spectrometer was equipped with a liquid nitrogen cooled CCD detector with optimal sensitivity in the visible (blue/green) and a chip size of 2,048 × 1,024 pixels (Symphony 3500, Jobin Yvon). The total spectral range (700–3,100 cm^{-1}) was recorded within 15 s of total integration time. Every measurement consisted of five averaged signal accumulations each with an actual exposure time of 3 s, and each sample was analyzed in triplicate. Toluene was used under the same conditions as an external standard for calibration by recording the position and the intensity of its well known symmetry ring breathing Raman band at 1,004 cm^{-1}. The baseline of each spectrum was approximated by a fourth-order polynomial fit in order to subtract the weak fluorescence background. Computer control of spectral recording and preprocessing was achieved using a commercial software (NGSLabSpec, Jobin Ivon).

Chemicals and Samples

All reagents used were of analytical grade. Oleic acid, diethylether, ethanol and potassium hydroxide (KOH) were purchased from Sigma-Aldrich. Oleic acid was used to expand the calibration interval for an increasing range of FFA content in one of the studied samples. Eighteen different brands of olive oil were purchased from a local grocery and one sample, produced using a traditional press, from a Tunisian farmer. All samples were stored at room temperature until the time of analysis.

Reference Analysis

The FFA was determined using the official method Ca 5a-40 approved by the American Oil Chemists' Society (AOCS) [23]. Briefly, a weighed sample of oil was dissolved into a mixture of solvents, a 1:1 volume ratio of ethanol and diethylether. Then, the mixture was titrated, under constant stirring, against a 0.1 M KOH solution using phenolphthalein as indicator. The results were presented as percentage oleic acid; the expression is given according to AOCS as

$$\%\ \text{FFA as oleic acid} = \frac{\text{Alkali volume (ml)} \times \text{Alkali normality} \times 28.2}{\text{Sample weight (g)}}$$

where 28.2 is the molecular weight of oleic acid divided by 10.

Chemometrics

For a chemometric analysis the spectra were exported from the NGSLabSpec software in text format to the Unscrambler software (v 9.7; CAMO A/S). Calibration models between reference data and Raman spectra were developed using a partial least squares (PLS) regression. Here, the data analysis included two separate steps, which were calibration and validation. In the calibration step, information relevant for the prediction was extracted from the Raman data in a few components while the validation step aimed to check whether the extracted components described the new data well enough. Cross validation was used in this approach, where one sample was left out from the calibration process and the remaining samples were used for calibration and then tested on the sample left out. The process was repeated until each of the samples had been used for validation once (left out once). The model accuracy was assessed by calculating the root mean square error

of the calibration (RMSEC) and the correlation coefficient (R^2) between actual and predicted values. The prediction ability of the model was assessed using the root mean square error of the validation (RMSEP) [24].

Results and Discussion

Raman Spectra

Raman spectra of different olive oils and oleic acid are shown in panels (a) and (b) of Fig. 1, respectively. Many major bands of the oleic acid were detected in the Raman spectra of the oil samples and are attributed to the main components in the oil, which are fatty acids. For example, the band at 1,265 cm^{-1} can be assigned to δ(=C–H) of *cis* R–HC=CH–R and the band at 1,300 cm^{-1} is characteristic of the C–H bending twist of the –CH_2 group, while the bands at 1,440, 1,650, and 1,750 cm^{-1} correspond to δ(C–H) scissoring of –CH_2, v(C=C) of *cis* RHC=CHR, and v(C=O) of RC=OOR, respectively. Furthermore, the bands at 2,850, 2,897, and 3,005 cm^{-1} are attributed to the symmetric CH_2 stretch, $v_s(CH_2)$, the symmetric CH_3 stretch, $v_s(CH_3)$, and the *cis* RHC=CHR stretch, v(=C-H), respectively [25]. The three bands, around 1,008 cm^{-1} (C-CH_3 bend), 1,150 cm^{-1} (C–C stretch), and 1,525 cm^{-1} (C=C stretch) are attributed to carotenoids [26, 27], which are responsible for the main characteristic variations in different brands of olive oil. The carotenoids in edible oils play a significant role as natural antioxidants. While these bands were not observed in previous studies using NIR excitation, they became prominently detectable under green excitation in our work and help to differentiate the differently produced olive oils.

Determination of the FFA Content

The FFA content of olive oil in terms of oleic acid percentage ranged from 0.14 to 0.40% as measured by the titration analysis. Pure oleic acid was used to expand the upper limit of the FFA range to 0.80%. Tunisian olive oil showed the highest FFA content, which could be due to the traditional way of handling, in which olives were stored in silos for long time leading to an increased enzymatic breakdown of the olive cell structure, especially if the fruit had been bruised during harvesting. The contact between olive and vegetation water—a fluidic waste resulting from olive oil extraction—is one of the factors of FFA increase in olive oil. The lower FFA content in manufactured olive oil reflects the care taken right from the storage of the olive fruits, through the production, up to the consumption of the oil.

The calibration model of FFA was constructed for nineteen different olive oils using PLS regression. The calibration curve of the actual FFA obtained by titration

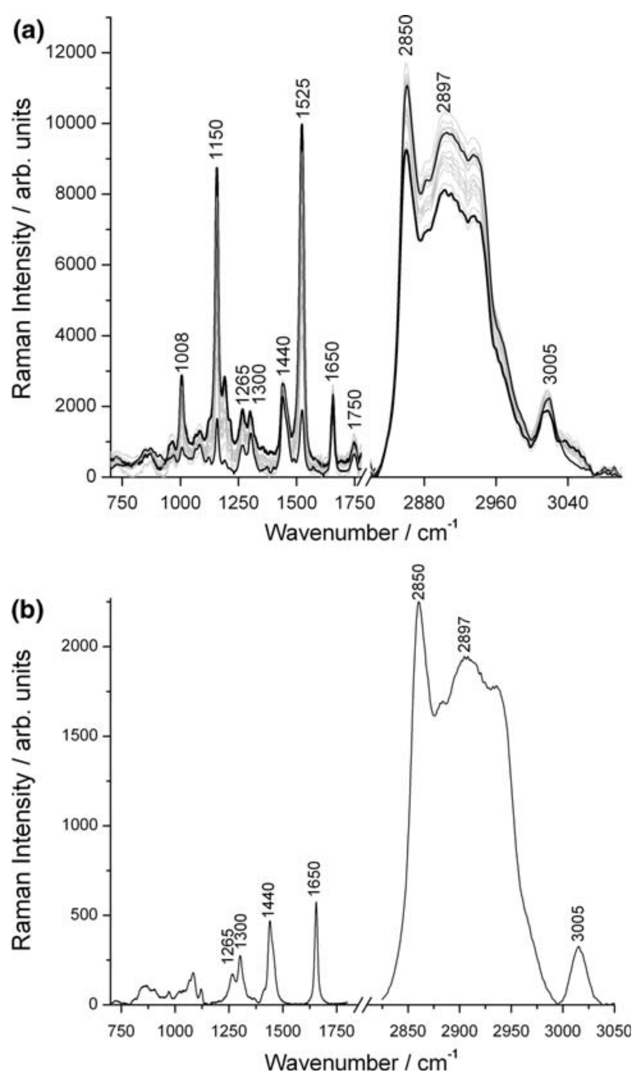

Fig. 1 Raman spectra of **a** 19 different virgin olive oil brands and **b** oleic acid. In **a**, the oils with the smallest and highest FFA contents are drawn as *black curves* (*upper* and *lower curve*, respectively). The other oil spectra are shown in *grey* to demonstrate the variation width of the Raman data

versus the predicted value was generated within a range between 0.14 and 0.80% of FFA. Firstly, quantification of the FFA level was achieved using the full Raman spectral window (700–3,050 cm^{-1}). However, it is important that the relevant spectral region includes characteristic information explaining the variation of the FFA concentration, since the inclusion of irrelevant spectral information yields an overfitting model. The relevant spectral regions were determined using PLS weighted coefficients (BW), which showed the relative importance of the spectral variables in the model (Fig. 2). Here, a positive coefficient shows a positive link with the FFA content, and a negative coefficient shows a negative link, while spectral variables with smaller coefficients are negligible. However, it is worth noticing that, in the case of a multidimensional data set, the

raw data are first represented within a reduced space of only few principal components (PCs) that describe the total variation within the data. In such a situation, the regression coefficients show the relative importance of the original variables in the plotted PC.

Figure 2 shows the weighted regression coefficients plotted for the first PC which accounts for a total variation of 90% within the samples. Interpreting the plot in this figure, we have found that the spectral fingerprint region (945–1,600 cm^{-1}) which also includes carotenoid bands is statistically significant in predicting the FFA % in olive oils, since this region yielded the largest regression coefficients. The bands at 1,650 cm^{-1} and the range (2,800–3,010 cm^{-1}) seem to be of relative importance as well, however, they were found to be less significant in this model, since their inclusion into the model led to the over-fitting of the model with a lower R^2 and a higher RMSE. We have constructed a new model based on a weighted regression analysis, using only the band at 1,650 cm^{-1} and the range (2,800–3,010 cm^{-1}). This yielded a poor model with a lower R^2 and higher RMSE as shown in Table 1. Nevertheless, this additional model might be of interest as a crosscheck of the results obtained from the first model, since carotenoid concentrations could be manipulated in order to e.g. hide attempts of fraud. Referring to panel (a) of Fig. 1, the change in carotenoid band intensity is noticeable. The olive oil with the lowest FFA content showed the highest intensity for the carotenoid bands (upper black curve), whereas the highest FFA content is associated with the lowest intensity of carotenoid bands (lower black curve). This peculiar situation could be due to carotenoid degradation caused by FFA. We have found that in previous works [28] the bleaching procedure to remove carotenoids from e.g. palm oil led to the increase of FFA. This points to a natural relationship between carotenoids and FFA in oils in general. Therefore, in order to improve the performance of the PLS model, the model was re-constructed using the selected significant spectral region.

Table 1 Statistical parameters of the PLS regression

Spectral range (cm^{-1})	R^2	RMSEC	RMSEV
700–3,050	0.505879	0.11695	0.1467
2,800–3,050 and 1,650	0.44803	0.2065	0.31763
945–1,600	0.963167	0.01193	0.034114

Table 1 shows, that using the whole Raman spectrum led to the over-fitting of the model performance as evidenced by the corresponding R^2s with FFA content and the RMSE for calibration and validation. In contrast to this, the region (945–1,600 cm^{-1}) yielded an improved and optimized model with an explained spectral variation of 98% for the first two components. This region is strongly influenced by the presence of carotenoid bands; a model constructed without the carotenoid bands was rather poor. This showed a strong correlation between FFA content and carotenoids. However, in other oil samples it is not excluded that other components might exist with more correlation to FFA than carotenoids.

Figure 3 shows the predicted FFA% based on Raman spectra versus the reference values, for the optimized model. The slope of the regression curve is close to 1, indicating a perfect linear relationship between predicted FFA values using Raman spectra and actual values using the titration method. This model showed higher R^2 and

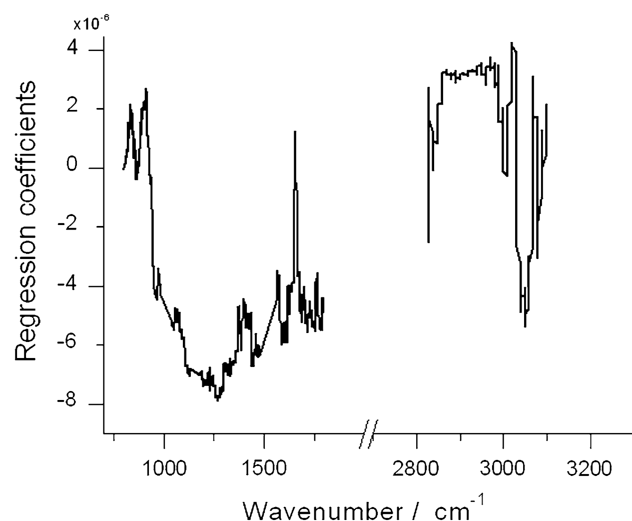

Fig. 2 Regression coefficient of Raman shift

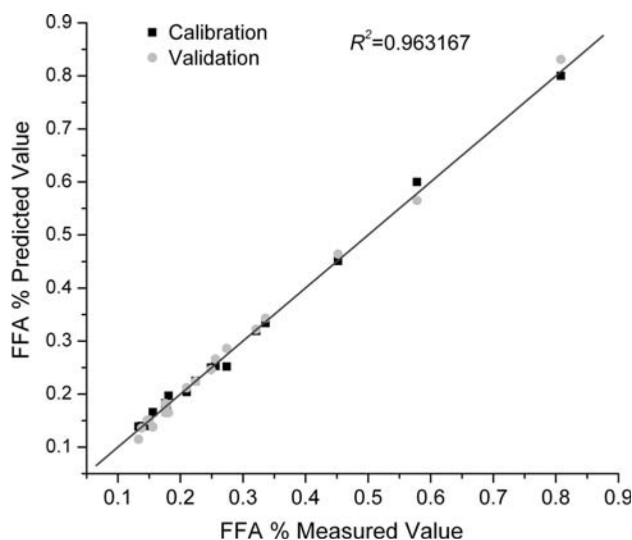

Fig. 3 Calibration curve of the FFA content values predicted using the Raman spectra versus the measured values using the standard titration method

lower RMSE for calibration and validation, which confirmed that the selected spectral range was the only significant region for the quantification of FFA. The equation used for the FFA content determination based on the constructed PLS model can be expressed as

$$FFA_p = 0.96\ FFA_t + 0.0031$$

where FFA_p is a predicted FFA concentration using PLS and FFA_t is a measured FFA concentration using the standard titration method.

In this work, the direct prediction of the content of FFAs in olive oil was successfully achieved using a rapid and non-distractive Raman spectroscopic method. The best optimization was achieved using the fingerprint region (945–1,600 cm^{-1}) of the Raman data, which yielded a robust and precise model with high accuracy. In our work the lowest value of FFA among all the samples measured was 0.14%. While this value is already comparable to the best achieved by other spectroscopic techniques, our detection limit could even be below, but we did not have among our samples one that was extremely pure (0% FFA) and in which we could add a smaller amount of oleic acid to find the lowest possible detection limit. These results demonstrated the potential of Raman spectroscopy for the determination of FFA content in olive oil under visible excitation. Minor species such as carotenoids, which were found to be statistically significant for the prediction of the FFA content, could be detected easily due to the resonance enhancement of their spectra. This technique can be implemented as a direct and rapid quality control technique of olive oil during both in-line production and distribution.

Acknowledgment We thank the Egyptian Ministry of Higher Education, Mission Department and the German Academic Exchange Service (DAAD) for their financial support (two Ph.D. fellowships).

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