Determination of Moisture in Olive Oil: Rapid FT-NIR Spectroscopic Procedure Based on the Karl-Fischer Reference Method

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Abstract: According to CODEX, moisture and volatile matter are olive oil quality parameters and the development of a rapid screening method for the determination of moisture is of interest. We recently demonstrated for the first time that the weak near-infrared (NIR) band near 5260 cm\(^{-1}\) is primarily attributed to a water O-H combination band. To determine the intensity of this band, we measured the peak-to-peak (p-p) height of its first derivative and generated exponential calibration curves for p-p height versus gravimetrically determined concentrations of spiked water in olive oils that had been purged of their initial moisture contents. To further optimize this univariate calibration method, calibration curves were generated in the present study based on plotting the moisture band first derivative p-p heights for neat olive oils (that were neither purged nor spiked) versus the moisture concentrations obtained by the Karl-Fischer (KF) primary reference method. To enhance the speed of FT-NIR data collection, measurements were carried in the transmission mode using disposable glass tubes. We also developed and compared a multivariate partial least squares approach to the univariate one. All the spectra were collected in two separate laboratories using two FT-NIR spectrometers of the same brand and model and no significant difference (\(p > 0.05\)) was found between the two laboratory determinations and the KF reference values at a 95% confidence interval. High accuracies were found with the two FT-NIR instruments used, as indicated by the low root mean squared error (RMSE, %) for predicted values obtained with the univariate procedure (RMSE = 0.008% and 0.010%) and the multivariate one, which yielded an even lower value (RMSE = 0.007% for both instruments). These results suggest that, once validated, the FT-NIR approach could potentially be a rapid substitute for the KF method.

Key words: moisture in olive oil, near-infrared spectroscopy, chemometrics, rapid screening

1 Introduction

The U.S. Food and Drug Administration (FDA) was directed by Congress to screen imported olive oils to determine if they are adulterated or misbranded\(^1\). Therefore, there has been a need to develop analytical methods that can rapidly determine olive oil purity and quality. According to CODEX, moisture and volatile matter are olive oil quality parameters and the recommended official method for the determination of all volatiles\(^2\) entails heating and measuring the loss in weight of an oil test portion\(^2\). The Karl-Fischer (KF) method is considered the gold standard for moisture determination and is sanctioned by officiating bodies such as AOCS and IUPAC\(^{3, 4} \). The KF method is time-consuming, relatively expensive to operate and maintain, and requires hazardous chemicals\(^3, 5\). Therefore, the development of chemical free rapid methods to determine moisture in olive oil is of interest since the concentration of moisture is potentially a marker for grade qualities of olive oil\(^6\). Spectroscopic methods are well suited for process-control applications because they are fast and can be easily implemented in-line or at-line. Among spectroscopic techniques, near-infrared (NIR) spectroscopy is generally

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selected because it is fast, non-destructive, requires little or no sample preparation and is suitable for on/in-line monitoring\(^7\). NIR spectroscopy in combination with chemometrics has been successfully applied to the quantitative analysis of moisture in various sample matrices such as compound fertilizers\(^7\), fish oils\(^8\), and intact olives\(^9\). Muik \textit{et al}. developed a NIR method to quantify oil and water contents in olive pomace\(^10\). Variation in the intensity of a weak NIR band near 5260 cm\(^{-1}\) was first observed for olive oil by Christy \textit{et al}.\(^11\). Recently, we demonstrated that this band is primarily attributed to a water O-H combination band\(^12\). To rapidly determine the concentration of moisture, a quality parameter according to CODEX\(^2\), test samples of olive oil used for calibration were purged with nitrogen and subsequently spiked with gravimetrically determined amounts of water\(^12\). The intensity of this water band was determined in the transfection mode by Fourier-transform (FT)-NIR by the univariate measurement of the peak-to-peak (p-p)/height of its first derivative. Exponential calibration curves for p-p heights versus gravimetrically determined concentrations of spiked water were generated and applied to the determination of moisture levels for a limited set of authenticated olive oils whose grades had been verified by official methods as well as for commercial products\(^12\).

In the present study, the accuracy of our previously developed NIR calibration procedure\(^12\) was investigated further. The moisture concentrations for 25 samples were determined by NIR and the KF method: 19 out of 25 olive oils evaluated by NIR had moisture contents below/above 80/120% of the corresponding values determined by KF method. We attribute this discrepancy primarily to the presence of a trace amount of residual moisture determined by KF that was found in the oil under our experimental conditions, which entailed purging and gravimetric spiking. This residual moisture may presumably have had a detrimental contribution to the accuracy of the quantitative determination of moisture in olive oil.

In the present study, our primary focus was to improve the accuracy of the calibration step in the FT-NIR spectroscopic procedure by using KF as the primary reference method. In the development of a quantitative calibration model by FT-NIR, the spectral information is related either to the known content of the analyte in the mixture obtained by a reference method, for instance based on gravimetric spiking or, in this case, the KF method\(^12\). Calibration curves were generated based on plotting the first derivative p-p heights of the weak near-infrared (NIR) band near 5260 cm\(^{-1}\) for olive oils (that were neither purged nor spiked) versus the corresponding KF method values. For evaluating the prediction performance of the newly developed calibration procedure, the generated calibration curves were then tested by using an independent test set of samples (n = 26) not used for calibration. We also developed and compared a multivariate partial least squares (PLS) approach to the results from the KF method. In addition, to avoid the time-consuming cleaning step of the transfection fiber optic probe after collecting spectra for each test sample\(^12\), to enhance the speed, and streamline data collection, FT-NIR measurements were carried out in the transmission mode using disposable glass tubes. To evaluate and compare the precision of the proposed analytical univariate and multivariate procedures, all spectra were collected in a limited collaborative study among two laboratories and subsequently analyzed.

2 Materials and Methods

2.1 Olive oil test samples

In order to measure a wide range of moisture concentrations by FT-NIR spectroscopy, we acquired and measured various grades of olive oils (n = 55) for calibration and validation. Their KF moisture concentrations were determined commercially (Eurofins QTA, Inc., West Chester, OH). A description of the chemical principle of the classical KF titrimetric moisture analysis is detailed in the AOCS Ca 2e-84 official method\(^13\). Briefly, in the coulometric KF method used\(^14\), the anodic compartment contains sulfur dioxide (SO\(_2\)), iodide (I\(^-\)) and imidazole (RN). Methanol (ROH) is used as a solvent. Iodine, which is generated from iodide, reacts with water in the oil according to the reaction scheme: The addition of RN to the methanolic solution shifts the equilibrium reaction to the right:

\[
\text{ROH} + \text{SO}_2 + \text{RN} \rightarrow (\text{RNH})\text{-SO}_4\text{R}
\]

and the KF reaction is expressed as:

\[
(\text{RNH})\text{-SO}_4\text{R} + 2\text{RN} + \text{I}_2 + \text{H}_2\text{O} \rightarrow (\text{RNH})\text{-SO}_4\text{R} + 2(\text{RNH})\text{I}
\]

The reaction is complete once all the water in the olive oil has reacted. The fifty-five oils used were obtained from different sources: 15 oils were obtained commercially from local stores in Montgomery County, MD, 22 were provided by the International Olive Council (IOC), and the remaining 18 were provided by the United States Pharmacopoeia (USP). This USP set of authenticated oils, whose quality and purity were verified with official methods, consisted of six extra virgin olive oils, four refined olive oils and eight pomace olive oils.

2.2 FT-NIR instrumentation

Two Bruker Optics model MPA FT-NIR spectrometers (Bruker Optics, Madison, WI) equipped with a thermoelectrically cooled indium gallium arsenide (InGaAs) detector were used for collecting spectra in the present limited collaborative study among two laboratories located at the FDA’s Center for Food Safety and Applied Nutrition (College Park, MD) and Bruker Optics (Madison, WI) facili-
ties. All test samples were measured at room temperature (22°C), in the transmission mode using 8-mm outer diameter (OD) glass disposable tubes (Bruker Optics). Spectra were collected by co-adding 32 scans at 8 cm⁻¹ resolution and by using the Blackman-Harris 3-term apodization function. For each test sample, duplicate test portions were used for data collection and measured in two different days. For the measurement of each test portion, triplicate spectra were collected and averaged, which yielded one average spectrum. For quantification, the absorbance spectrum collected over the entire spectral range 4,500-10,000 cm⁻¹ was converted to its first derivative (smoothing points = 9) and the p-p height of the first derivative of the band near 5260 cm⁻¹ was measured by adding the absolute values of the maximum and minimum intensity amplitudes of the positive and negative first derivative peaks, respectively.

2.3 Chemometrics data analysis

Multivariate data analysis based on partial least squares regression (PLSR) was performed by using PLS_Toolbox chemometrics software (PLS_Toolbox_8.0.1, Eigenvector Research Inc., Wenatchee, WA, USA) that runs in a MATLAB computational environment (MATLAB 8.3, The Mathworks Inc., Natick, MA, USA). The relationships between the actual concentrations of moisture in a test sample, as determined by KF analysis and the corresponding observed FT-NIR spectra were established by applying PLSR analysis. PLSR calibration models were developed and optimized for data collected with each of two FT-NIR benchtop spectrometers.

The optimum number of PLS factors for the calibration model was selected based on evaluating the root mean square error of cross-validation (RMSECV) given by:

$$\text{RMSECV} = \sqrt{\frac{1}{N} \sum_{i=1}^{N} (y_i - \hat{y}_i)^2}$$

Where, $y_i$ is the measured value for moisture by the KF method, $\hat{y}_i$ is the value predicted by PLSR of $i^{th}$ test sample, and $N$ is the number of test samples in the calibration set.

To find the optimum number of factors for each calibration model, the RMSECV values were plotted against the number of PLS factors. The number of factors that yielded a minimal RMSECV value was considered to be optimal and subsequently used to build a calibration model. Including too many factors in a model or overfitting would deteriorate the prediction ability for the test samples in a test set. Several PLSR calibration models were developed using different data pre-processing techniques and for different spectral regions that included the moisture spectral feature near 5260 cm⁻¹. The optimal range used was 4500-6000 cm⁻¹ for developing PLS calibration models. Different spectral pre-processing techniques were also evaluated to correct for the baseline effects found around 5260 cm⁻¹, and possibly for the presence of potentially overlapping weak interference bands. The spectral pre-processing techniques tested were Savitzky-Golay first derivatives, multiplicative scatter correction (MSC), or the combination of MSC and Savitzky-Golay first derivatives. The optimal spectral pre-processing method and spectral region combination were selected based on the minimum RMSECV value.

2.4 Statistical analysis

Moisture concentrations calculated from the two FT-NIR and KF methods were compared using parametric paired t-test. Prior to the t-test, the assumption of normality was evaluated by using the Shapiro–Wilk test [15]. Statistical analyses for paired t-tests calculations and to test the assumption of normality were performed using JMP (version 11.2.0; SAS Inst., Cary, NC, U.S.A). A comparison of the moisture determinations by FT-NIR and KF was performed according to the procedure of Bland and Altman [16]. The

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Fig. 1 Expanded FT-NIR spectral region that exhibits the observed differences in the intensity of (a) the band near 5260 cm⁻¹ for several representative olive oils, and (b) their corresponding first derivative spectra. Observed variation in band intensity is primarily due to differences in moisture concentration.
3 Results and Discussion

The principal objective of this work has been limited to developing rapid univariate and multivariate FT-NIR procedures for determining moisture concentration in olive oils with the highest possible accuracy. Recently we attempted to achieve this task by using calibration test samples for olive oil that had been purged with dry nitrogen and subsequently spiked with gravimetrically determined amounts of water\(^\text{12}\). However, the resulting calibration curves yielded unsatisfactory determinations relative to the KF method. These results were attributed mostly to the presence of a trace amount of residual water that was present in the olive oil under our experimental conditions. In the present study, we sought to improve the accuracy of the FT-NIR procedure by generating univariate calibration curves based on plotting the first derivative p-p heights of the band near 5260 cm\(^{-1}\) observed for pure olive oils that have not been purged with nitrogen gas or spiked with water versus the KF method values.

**Figure 1a** illustrates the FT-NIR O-H combination moisture band for a representative number of test samples taken from the 29 olive oils used for calibration in the present study. Their moisture concentration range included the maximum and minimum values found by the KF method, which fell in the range of 0.023% to 0.107% (w/w). Because the observed band near 5260 cm\(^{-1}\) observed for pure olive oils (that have not been purged with nitrogen gas or spiked with water) versus the KF method values.

**Figure 2** exhibits the calibration curves for these 29 test samples. The plots display the p-p first derivative moisture band intensities as a function of the KF moisture concen-

![Fig. 2](image)

**Fig. 2** Calibration curves generated for peak-to-peak first derivative band heights of the band near 5260 cm\(^{-1}\) versus moisture concentrations determined with the gold standard Karl-Fischer method for spectra collected with two different FT-NIR spectrometers of the same brand and model at (a) FDA, and (b) Bruker Optics facilities.

Bland–Altman approach was used, as an alternative to the correlation coefficient, to statistically compare the performance of the two orthogonal analytical methods for the determination of one or more analytes.

**Fig. 3** Bland–Altman plots comparing moisture concentrations determined with the gold standard Karl-Fischer primary method to those predicted by a univariate FT-NIR procedure using spectra collected with two different FT-NIR spectrometers at (a) FDA, and (b) Bruker Optics facilities. The differences between two determined pairs obtained by FT-NIR and KF are plotted against their means. Each point represents a single test samples from the independent set of 26 olive oils used for prediction.
Near-Infrared Determination of Moisture in Olive Oil

Given the unusually narrow moisture concentration range (0.023-0.107 %, w/w) found for the set of 29 olive oils used for calibration, the RF values, which were similar, 0.83 (Fig. 2a) and 0.81 (Fig. 2b), were acceptable for the rapid screening of moisture concentrations.\(^7\)

Moisture concentrations, determined by using this univariate procedure based on data collected with two different FT-NIR spectrometers and by the KF method, for an independent set of 26 test samples (not used for calibration) are compared in Fig. 3 and Table 1. All determinations were carried out in duplicate over two days and averaged. Excellent agreement in moisture determination among the two FT-NIR instruments and the KF method was found. When the parametric paired t-test was performed for method comparison, \(p > 0.05\) were obtained, which indicated that no significant difference exists between the two FT-NIR data sets and the KF method at a 95% confidence interval. Prior to performing the t-test, the assumption of

### Table 1
Comparison of moisture concentrations (% w/w) for a test set of 26 olive oils determined by a univariate and predicted by a multivariate FT-NIR spectroscopic procedures with those obtained by using the Karl-Fischer method. FT-NIR spectra were collected in two laboratories using two Bruker MPA spectrometers at FDA and Bruker Optics laboratories.

| Sample | Reference KF | FT-NIR (FDA), Univariate * | FT-NIR (Bruker Optics), Univariate * | FT-NIR (FDA), Multivariate * | FT-NIR (Bruker Optics), Multivariate * |
|--------|--------------|---------------------------|-------------------------------------|----------------------------|-------------------------------------|
| 1      | 0.027±0.001  | 0.023±0.000               | 0.019±0.000                         | 0.025±0.000                | 0.025±0.000                         |
| 2      | 0.030±0.001  | 0.037±0.000               | 0.037±0.000                         | 0.035±0.000                | 0.035±0.000                         |
| 3      | 0.042±0.001  | 0.046±0.000               | 0.045±0.000                         | 0.057±0.000                | 0.056±0.001                         |
| 4      | 0.046±0.000  | 0.045±0.000               | 0.045±0.000                         | 0.054±0.000                | 0.053±0.000                         |
| 5      | 0.047±0.001  | 0.052±0.000               | 0.054±0.001                         | 0.046±0.000                | 0.046±0.001                         |
| 6      | 0.048±0.001  | 0.056±0.000               | 0.055±0.000                         | 0.050±0.000                | 0.048±0.000                         |
| 7      | 0.049±0.002  | 0.055±0.000               | 0.054±0.001                         | 0.050±0.000                | 0.049±0.000                         |
| 8      | 0.050±0.002  | 0.056±0.000               | 0.054±0.000                         | 0.050±0.000                | 0.048±0.001                         |
| 9      | 0.052±0.006  | 0.053±0.000               | 0.054±0.000                         | 0.045±0.001                | 0.046±0.000                         |
| 10     | 0.053±0.003  | 0.056±0.000               | 0.054±0.001                         | 0.049±0.000                | 0.049±0.001                         |
| 11     | 0.053±0.000  | 0.057±0.000               | 0.055±0.001                         | 0.055±0.000                | 0.053±0.001                         |
| 12     | 0.055±0.001  | 0.060±0.000               | 0.063±0.001                         | 0.058±0.000                | 0.059±0.001                         |
| 13     | 0.055±0.001  | 0.052±0.000               | 0.055±0.001                         | 0.060±0.000                | 0.062±0.000                         |
| 14     | 0.057±0.001  | 0.061±0.000               | 0.055±0.000                         | 0.060±0.000                | 0.056±0.000                         |
| 15     | 0.061±0.003  | 0.079±0.000               | 0.083±0.000                         | 0.063±0.001                | 0.064±0.000                         |
| 16     | 0.062±0.002  | 0.067±0.000               | 0.062±0.000                         | 0.067±0.001                | 0.063±0.000                         |
| 17     | 0.065±0.006  | 0.062±0.000               | 0.055±0.000                         | 0.060±0.000                | 0.057±0.000                         |
| 18     | 0.066±0.005  | 0.087±0.000               | 0.093±0.000                         | 0.075±0.000                | 0.079±0.000                         |
| 19     | 0.071±0.001  | 0.078±0.000               | 0.081±0.001                         | 0.075±0.001                | 0.077±0.000                         |
| 20     | 0.073±0.003  | 0.083±0.000               | 0.057±0.000                         | 0.080±0.000                | 0.062±0.001                         |
| 21     | 0.076±0.003  | 0.071±0.000               | 0.076±0.000                         | 0.074±0.000                | 0.073±0.001                         |
| 22     | 0.077±0.001  | 0.081±0.000               | 0.082±0.000                         | 0.072±0.000                | 0.071±0.000                         |
| 23     | 0.078±0.007  | 0.077±0.000               | 0.079±0.001                         | 0.063±0.001                | 0.063±0.000                         |
| 24     | 0.082±0.002  | 0.083±0.000               | 0.084±0.000                         | 0.083±0.001                | 0.083±0.000                         |
| 25     | 0.084±0.010  | 0.076±0.000               | 0.079±0.000                         | 0.068±0.000                | 0.070±0.000                         |
| 26     | 0.088±0.006  | 0.107±0.000               | 0.111±0.000                         | 0.090±0.000                | 0.092±0.000                         |

*Values are reported as mean of duplicate measurements ± the difference between each duplicate measurement from their mean.

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normality was evaluated by using the Shapiro–Wilk test and all data sets were found to be normally distributed. High accuracies were found with the two FT-NIR instruments used, at FDA and Bruker Optics, as indicated by the low root mean squared error of prediction (RMSEP, 0.0088%, and 0.010%, respectively) for moisture concentrations determined by this univariate procedure.

To further compare the performance of these two orthogonal analytical methodologies, the Bland–Altman method was used (Figs. 3a and 3b). The differences between two determined pairs obtained by FT-NIR and KF are plotted against their means. Agreement between methods is assessed in terms of the bias of the mean, the size of the 95% confidence interval (i.e., ±2 SD), and the spread of data points within the 95% confidence interval. The limits of agreement between FT-NIR and KF are defined by the mean difference ±2 SD of the mean differences obtained for all the test samples analyzed. In the present study, when the predicted values for the data collected with the FT-NIR spectrometer at FDA were compared with the KF values, all points fell within the ±2 SD limits, except for three test samples (Fig. 4a). Similarly, for the FT-NIR data from the Bruker Optics laboratory, three samples fell outside the ±2 SD limits (Fig. 3b). Two common samples were found to fall outside the ±2 SD limits for both instruments. The samples that were outside the mean ±2 SD limits were however located close to these limits (Figs. 3a and 3b). For the two FT-NIR spectrometers used, the mean differences for moisture concentrations were low, −0.005% and −0.004% (w/w), respectively, indicating a satisfactory degree of similarity in analytical determination.

To explore the potential advantage of using a multivariate data analysis approach for predicting moisture concentration, we also evaluated the performance of FT-NIR spectroscopy in conjunction with PLSR. This procedure was developed in order to minimize the error associated with quantification of the weak, overlapping moisture absorption band near 5260 cm⁻¹ that is not baseline-resolved. Models were developed by using the calibration set of samples and were validated by using the calibration-independent set of 26 test samples.

The optimized PLSR calibration model parameters based on cross-validation of the FT-NIR data are described in terms of the number of PLS factors, spectral regions, and spectral preprocessing methods, and as evidenced by the smallest RMSECV. The optimum models were developed with two PLS factors for the multiple scatter correction (MSC) preprocessed spectral data in the 4500-6000 cm⁻¹ range for both FT-NIR instruments. PLS-predicted moisture concentrations for 26 test samples in this independent data set used for validation (Table 1) indicated excellent agreement among the data sets for nearly all the test samples. As previously stated, when the parametric paired t-test was performed for the method comparison, p-values > 0.05 were obtained indicating no significant difference between the multivariate predictions obtained for spectra collected with the two FT-NIR instruments and the determinations based on the KF method. High accuracies were found with the two FT-NIR instruments as indicated by the low root mean squared error of prediction (RMSEP, 0.007% for both instruments). To further compare the performance of these two orthogonal analytical methodologies, the Bland–Altman method was used (Figs. 4a and 4b). Two common samples were found to be outside the ±2 SD limits for both instruments. The samples that were outside the mean ±2 SD limits were located close to these limits (Figs. 4a and 4b). The differences between the KF-determined and PLSR-predicted pairs are plotted against their means. Each point represents a single test sample from the independent set of 26 olive oils used for prediction.

**Fig. 4** Bland–Altman plots comparing moisture concentrations determined with the gold standard Karl-Fischer primary method to those predicted by a multivariate FT-NIR/PLSR procedure using spectra collected with two different FT-NIR spectrometers at (a) FDA, and (b) Bruker Optics facilities. The differences between two determined pairs obtained by FT-NIR/PLSR and KF are plotted against their means. Each point represents a single test sample from the independent set of 26 olive oils used for prediction.
lytical determination.

No significant difference was observed between the moisture concentrations obtained with the FT-NIR univariate and multivariate procedures \( (p>0.05) \), however the former approach is preferred since it requires less expertise in calibration model development and the use of relatively fewer calibration test samples for achieving optimal determinations. By contrast, the development and validation of a successful multivariate quantitative modeling tool, such as the supervised PLSR, in conjunction with vibrational spectroscopic data requires expertise and involves several steps: These include selecting a training set consisting of representative reference olive oil that account for the possible variability present in test samples. In other words, it requires the availability of a large number of representative genetic varieties of olive oil samples from many countries and geographic regions, and selecting an appropriate reference method (such as KF), since the robustness of a calibration model will be dictated by its accuracy and precision; performing signal preprocessing to remove extraneous sources of variance (such as baseline shift) that are irrelevant to the analysis; building a multivariate calibration model; optimizing the number of factors; validating the model; and applying it to the determination of moisture concentration.

4 Conclusions

A rapid and simple FT-NIR procedure to determine moisture concentration in olive oil was developed. The use of KF as the primary reference method during the calibration step minimized the introduction of potential experimental errors and enhanced the accuracy of the FT-NIR determination. The use of disposable glass tubes in the transmission mode eliminated the potential for cross contamination from the use of a fiber optic probe in the transmission mode which eliminated the potential for cross contamination by using disposable glass tubes in the transmission mode. No significant difference \( (p>0.05) \) was observed between the FT-NIR and the KF moisture determinations at a 95% confidence interval, which suggests that the former, which provides an obvious time- and cost-saving alternative to the KF method and would be acceptable for routine screening applications. This proposed procedure should be applicable to other edible fats and oils as long as their NIR spectral fingerprints are similar to those of the various grades of olive oils and exhibit the moisture band near 5260 cm\(^{-1}\).

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