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Rapid quantitative determination of oleuropein in olive leaves (*Olea europaea*) using mid-infrared spectroscopy combined with chemometric analyses

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ABSTRACT

Oleuropein, the major active compound in olive leaf, is well known for its benefits for human health. Oleuropein is classically quantified by HPLC, which is time and chemical consuming, laborious and expensive. The aim of this work was to examine the potential of mid-infrared spectroscopy, as a rapid tool, to predict oleuropein content in olive leaf from five Tunisian cultivars (*Chemlali*, *Chetoui*, *Meski*, *Sayali* and *Zarrazi*) and one French cultivar (*Bouteillan*). The reference data of oleuropein content were obtained by the HPLC method. Hundred five samples were analyzed by HPLC and mid-infrared spectroscopy. Samples were randomly divided in a calibration set (73 samples) and in a validation set (32 samples). The spectral data sets were correlated with reference data of oleuropein content by using partial least squares (PLS) regression algorithm. The results showed that the PLS model gave satisfactory model for quantitative prediction of oleuropein content in olive leaf (relative error of prediction = 8.5%). The correlation coefficient was 0.91 and 0.74 for calibration set and validation set, respectively. It can be concluded that mid-infrared spectroscopy constitutes a promising technique for rapid quantification of oleuropein in olive leaf.

1. Introduction

Oleuropein (Fig. 1), a secoiridoid compound, is present in the whole *Olea europaea* L. olive tree and its derivatives (olive oil, olive mill wastewater and pomace). It is the most abundant biophenol and the major bioactive compound in olive leaves. Olive leaves, resulting with huge amount from pruning or defoliation of olive fruits before processing, were showed be useful source for oleuropein extraction (Savournin et al., 2001; Bouaziz and Sayadi, 2003; Jàpon-Lujan et al., 2006).

It has been reported that oleuropein possesses many beneficial effects on human health, such as antioxidative (Benavente-Garcia et al., 2000), antimicrobial (Pereira et al., 2007), antiviral (Micol et al., 2005), anti-ischemic (Andreadou et al., 2006), anti-inflammatory (Visioli et al., 1998) and hypolipidemic (Jemai et al., 2008) properties. In addition, oleuropein has shown cardioprotective (Andreadou et al., 2006) and neuroprotective (Omar, 2010) effects. In vitro studies have demonstrated that oleuropein acts as an antitumor compound (Hamdi and Castellon, 2005), inhibits platelet-activating factor activity (Andrikopoulos et al., 2002) and might be a modulator of metabolism. It improves lipid metabolism to protect against obesity problems (Polzonetti et al., 2004). Furthermore, oleuropein intervenes in the developmental processes of olive fruits and tree. It also defends olive tree against the attack of pathogens and insects (Malik and Bradford, 2006).

The quantification of oleuropein in olive leaves is usually carried out using high performance liquid chromatography (HPLC) (Savournin et al., 2001; Jàpon-Lujan et al., 2006; Ranalli et al., 2006). High-resolution gas chromatography (HRGC) has also been used (Ranalli et al., 2006). These analytical techniques are time-consuming, expensive and involve a considerable amount of manual work. Very often, complex chemical treatment of the sample and the use of sophisticated instruments are required.

Recently, mid-infrared (MIR) spectroscopy has become an emerging well-accepted analytical technique, due to its simplicity with advantages in terms of cost per sample. It achieves high analysis speed and requires little or no sample preparation. MIR spectroscopy has been widely used as an analytical tool in

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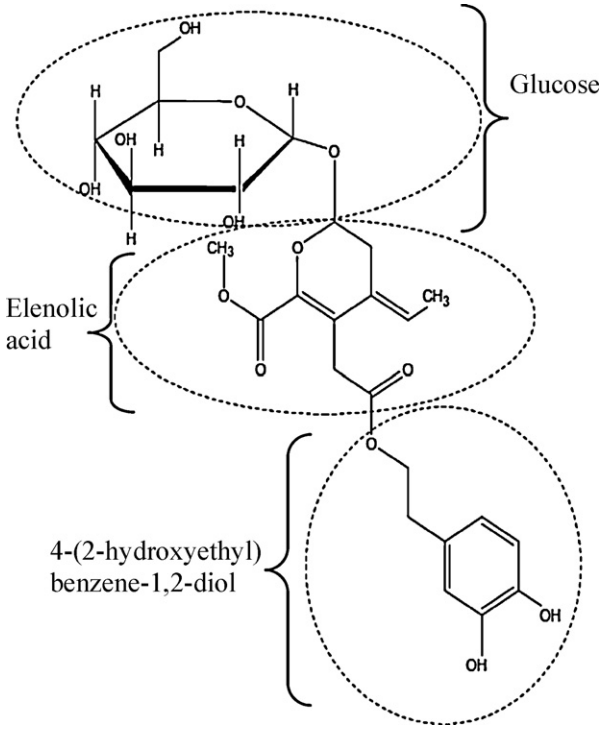

Fig. 1. Molecular structure of oleuropein.

various laboratories and industrial sectors such as food agricultural (Irudayaraj and Tewari, 2003; Käuper and Ferri, 2004; Bureau et al., 2009), pharmaceutical (Wu et al., 2008), petrochemical (Galtier et al., 2011), geochemical (Reeves and Smith, 2009), planetary and space science (Kelley and Wooden, 2009). Up to date, a lot of studies have been published on the utilization of MIR spectroscopy for predictive quantification of constituents in many agro-foods, such as lipid composition in virgin olive oils (Galtier et al., 2008); polymethoxylated flavone content in orange oil residues (Manthey, 2006); sugars and organic acids contents in apricot fruit (Bureau et al., 2009), in apple juice (Irudayaraj and Tewari, 2003) and in tomatoes (Beullens et al., 2006); total phenol, total flavonoids, total anthocyanin and ascorbic acid content in blueberries (Sinelli et al., 2008); residual starch from in vitro fermentations (Udén, 2009). However, to our knowledge, no work with quantitative approach has been conducted on olive leaves by application of MIR spectroscopy.

The aim of this study was to develop by mid-infrared spectroscopy associated to chemometric treatment, a direct and rapid tool to quantitative determination of oleuropein in olive leaves.

2. Materials and methods

2.1. Chemicals

Oleuropein standard was obtained from Extrasynthèse (Geney, France). Formic acid and methanol of HPLC grade were purchased from Sigma–Aldrich.

2.2. Olive leaf samples

Experiments were carried out on 105 samples of olive leaves from six cultivars (*Chemlali* (A), *Sayali* (B), *Meski* (C), *Zarrazi* (D) and *Chetoui* (E) from Tunisia and *Bouteillan* (AL) from France). All Tunisian trees were cultivated in the same experimental field and cultural conditions of the collection of olive trees varieties (RESGE CFC/COI/03) – Oued Souhil – Nabeul 2004 (Tunisia). Bouteillan cultivar trees were cultivated in Saint-Cyr-sur-Mer (France). Sampling from French trees was done in January 2010 as following: young leaves (coded A1L) were harvested from the fruiting zones on small branches (diameter < 2 mm) and mature leaves (coded A2L) were harvested from large branches (diameter > 4 mm). Young leaves from Tunisian trees were collected in October 2009. For each of the 6 cultivars, the samples were obtained from 3 different trees (coded (1), (2) and (3)). From each Tunisian tree, leaves were harvested from the 5 orientations of tree (Centre (C), North (N), South (S), East (E) and West (W)). In all cases, every sample contains about 100 healthy leaves. Tunisian samples were dried on site in a microwave two times for 2 min at maximum power 800 W (2450 MHz). French samples were lyophilized. Dried leaves were powdered in a blender IKA Labortechnik A10 and stored at 4 °C in the dark until use.

2.3. Extraction of oleuropein from olive leaves

Powdered samples (1 g) were extracted by 30 mL of methanol/water (4/1, v/v) for 24 h under agitation. The resulted extracts were centrifuged at 4500 rpm for 15 min. The supernatants were recovered and used for HPLC analyses.

2.4. HPLC determination of oleuropein content

HPLC analyses were performed with an analytical HPLC unit (Agilent technologies 1200 series), equipped with a diode array detector. The stationary phase was an Atlantis® Waters dC18 column (5 μm particle size; 250 mm; 4.6 mm). The mobile phases were formic acid (19:1) (A) and methanol (B), starting with 5% B and installing a gradient to obtain 15% B at 3 min, 25% B at 13 min, 35% B at 25 min, 45% B at 35 min, 50% B at 40 min, 100% B at 45 min, 5% B at 46 min, finally, reequilibration in 4 min to initial composition. The flow rate was 0.9 mL min^{-1} with elution at room temperature. The injection volume was 10 μL and chromatograms were recorded at 280 nm. The data were processed on the ChemStation Agilent technologies software. Oleuropein in olive leaf extract was identified by matching the retention time and the UV spectra of a peak in the extract chromatogram with the peak of the oleuropein standard. Identifications were confirmed by analyzing a sample supplemented by the oleuropein standard.

2.5. Mid-infrared analyses

2.5.1. Instrumentation

Mid-infrared spectra of samples were obtained using a Thermo Nicolet AVATAR 370 FT-IR spectrometer equipped with a deuterotriglycine sulfate (DTGS) detector, an Ever-Glo mid-infrared source and a KBr/germanium beam splitter. The infrared spectrometer was situated in an air-conditioned room (21 °C).

2.5.2. Analysis conditions

Dried powdered olive leaf samples (30–50 mg) were analyzed by mid-infrared spectroscopy. Samples were deposited on attenuated total reflection (ATR) cell equipped with a diamond crystal prism (monoreflexion). Mid-infrared spectra were recorded between 4000 cm^{-1} and 700 cm^{-1}. The nominal resolution was 4 cm^{-1}, and 100 scans were co-added. Air was taken as reference for the background spectrum before collection of each sample spectrum. Between each spectrum, the ATR plate was cleaned in situ by scrubbing with ethanol solution, which made it possible to dry the ATR. Cleanliness was verified by collecting a background spectrum and comparing it to the previous background spectrum. Each sample was scanned with three replicates. The scans of each sample were

examined visually for consistence, and the average spectrum of each sample was used for further analyses.

2.6. Chemometric treatments of MIR spectra

The MIR data were transformed with standard normal variable (SNV) and first derivative to remove slope variation and to correct for scatter effects. The partial least square (PLS) regression method was used to develop models for predicting the oleuropein content in olive leaf. PLS (Fuller and Griffiths, 1978; Haaland and Thomas, 1988) was initially built for quantitative analysis, but now it is also used for pattern recognition. This supervised analysis is based on the relation between spectral intensity and sample characteristics (Martens and Naes, 1989). Interference and overlapping of the spectral information may be overcome using powerful multicomponent analysis such as PLS regression. The ability of this algorithm is to mathematically correlate spectral data to a property matrix (Dupuy et al., 2005). Mean centering is applied before modeling. The number of latent variables selected for the PLS model was obtained by cross validation on the calibration set.

The whole spectral collection included 105 spectra, each corresponding to one sample. In order to carry out a cross-validation test, the data set was randomly divided into two groups: two third of the samples ($n = 73$) were used for calibration and one third ($n = 32$) was used for validation. The model was built by the cross validation method during the calibration developments. The evaluation of the errors in the calibration was carried out by computing the standard error of calibration (SEC) after comparing the real oleuropein content with the computed one for each sample. The formula for the standard error of calibration is:

$$SEC = \sqrt{\left(\frac{\sum_{i=1}^{N}(C_i - C_i')^2}{N - 1 - p}\right)} \quad (1)$$

where C_i is the known value, C_i' is the value calculated by the calibration equation, N is the number of samples, and p is the number of independent variables in the regression optimized by cross-validation.

The standard error of prediction (SEP) gives an estimation of the prediction performance during the step of validation of the calibration equation:

$$SEP = \sqrt{\left(\frac{\sum_{i=1}^{M}(C_i - C_i')^2}{M}\right)} \quad (2)$$

where M is the number of samples in the prediction set.

The root mean square errors of calibration (RMSEC) and of prediction (RMSEP) were also calculated. The two values were obtained using cross validation and they were expressed in the same units as the original response values. RMSEC can be interpreted as the average calibration error and RMSEP can be interpreted as the average prediction error.

Another useful parameter is the relative error of prediction (REP), which shows the predictive ability of the model, calculated from the equation

$$REP = \frac{SEP}{\bar{y}} \times 100 \quad (3)$$

where $\bar{y}$ is the mean of the observed values of crop variables.

Acceptable models should have low SEC, RMSEC, SEP, RMSEP and REP, and high coefficient of correlation between predicted and reference value.

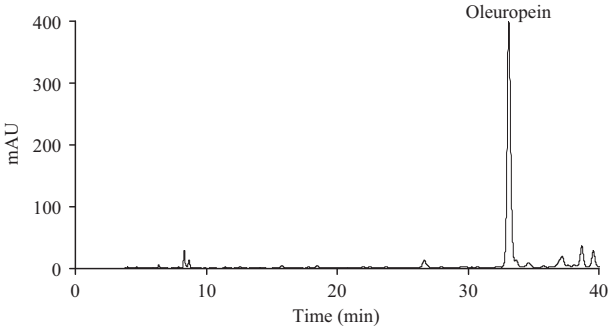

Fig. 2. HPLC chromatogram of an extract of olive leaf sample. Peak oleuropein ($t_R = 33.07$ min). Detection was at 280 nm.

2.7. Software for MIR data treatments

FTIR-ATR spectra have been recorded by the instrument software OMNIC 4.1b (Thermo Nicolet). UNSCRAMBLER software the version 9.6 from CAMO (Computer Aided Modelling. Trondheim. Norway) has been used for chemometric treatment of FTIR-ATR data spectra.

3. Results and discussion

3.1. Reference data of oleuropein content

The reference data of oleuropein content in olive leaves were obtained by the classical technique of HPLC (Savournin et al., 2001; Bouaziz and Sayadi, 2005; Jàpon-Lujan et al., 2006). The oleuropein was eluted at $t_R = 33.07$ min (Fig. 2). It was found to be the major phenolic compound in olive leaves as reported by many authors (Benavente-Garcia et al., 2000; Bouaziz and Sayadi, 2005; Jàpon-Lujan et al., 2006).

The calibration curve of oleuropein standard was linear ($R^2 = 1.000$) in the amount range from 4 to 120 μg. The least-square regression line was: $Y = 0.0032X$, where Y is the mass of the oleuropein standard (μg) and X is the area of corresponding peak. The linearity of the HPLC method was also studied by injecting various volumes of an olive leaf extract. The plotted curve, correlating the peak area of oleuropein in olive leaf extract versus the corresponding injected volume, was linear ($R^2 = 0.9998$) in the volume range from 2 to 20 μL. The least-square regression line was: $Y = 1190.1X - 212.37$, where Y is the area of oleuropein peak and X is the injected volume (μL).

Values of oleuropein content, expressed in percentage (w/w) (gram oleuropein per 100 g dry matter of olive leaves), ranged between 8.72% and 17.95% as shown in Table 1. These data confirm why olive leaves are a source of oleuropein (Savournin et al., 2001; Bouaziz and Sayadi, 2003; Jàpon-Lujan et al., 2006). The obtained oleuropein contents were within the range of levels reported by Bouaziz and Sayadi (2005). They got 12.4–14.2% on a dry mass basis, depending to the harvesting time. Similarly, Savournin et al. (2001)

Table 1
Oleuropein content in both calibration and validation sample sets of olive leaves.

	Calibration set (g/100 g DM[a])	Validation set (g/100 g DM)
Numbers	73	32
Min	8.72	10.92
Max	17.95	17.17
Mean	13.61	13.70
Median	13.31	13.79
Standard deviation	1.90	1.71

[a] DM, dry matter.

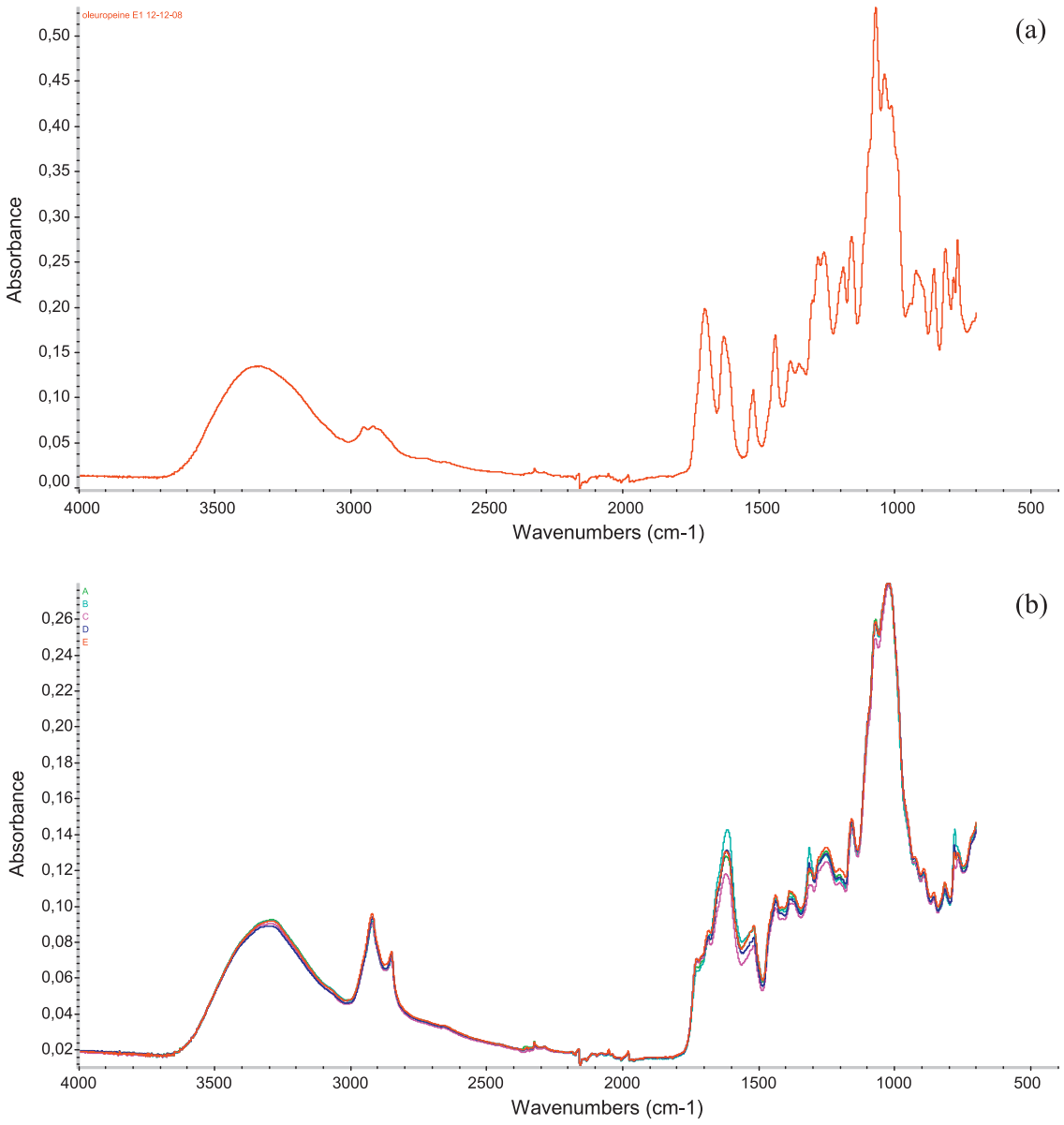

Fig. 3. MIR spectrum of oleuropein standard (a) and MIR spectra of various powdered olive leaf samples (b).

found about 9–14.3% of oleuropein for 14 cultivars. However, values obtained in our study were higher than reported by Jemai et al. (2008) who only extracted 4.32 g of oleuropein per 100 g dry matter of olive leaves. Also, Jàpon-Lujan et al. (2006) only extracted up to 23 mg of oleuropein per 1 g of dried olive leaves.

The descriptive statistic for the reference data of oleuropein content in both calibration and validation sample sets of olive leaf samples are shown in Table 1. The ranges and standard deviations for oleuropein content in both calibration and validation sets indicate high diversity in this parameter. It could be due to genetic diversity (6 cultivars), drying process (lyophilization and microwave) and/or harvesting time (October and January) of olive leaf samples. According to Table 1, the small differences in means (13.61% and 13.70%), median (13.31% and 13.79%), range (8.72–17.95% and 10.92–17.17%) and standard deviation (1.90% and 1.71%) between the calibration and the validation sets indicated that both sets represented the whole variation of all the olive leaf samples used. Therefore, the distribution of the samples is appropriate both in the calibration and in the prediction sets.

3.2. Spectral analyses

MIR spectrum performed for oleuropein was shown in Fig. 3a and has similarities with the spectra of dried olive leaves. Typical average spectra from leaves of each cultivar were presented in Fig. 3b. MIR analysis was performed on homogenate samples obtained by milling a lot of dried olive leaves. The MIR spectra obtained for all samples were similar by visual inspection, indicating that no noticeable qualitative difference was existed between leaf samples. All spectra are characterized by common absorption bands. However, the spectra present some weak differences between 1750 and 700 cm^{-1} in the relative intensities of absorbance from various cultivars. A broad band (3700–3000 cm^{-1}) is due to OH stretching vibrations (νOH) (oleuropein, cellulose, organic acids, etc.) and the two bands (3000–2800 cm^{-1}) from symmetric and asymmetric CH stretching vibrations (νCH, alkyl). The 1800–1500 cm^{-1} region corresponds to C=O and C=C stretching vibrations (esters, acid, carboxylate, aromatic ring). The 1500–1200 cm^{-1} range is very complex with especially CH (δCH, alkyl), and OH deformation vibrations as CO stretching vibrations

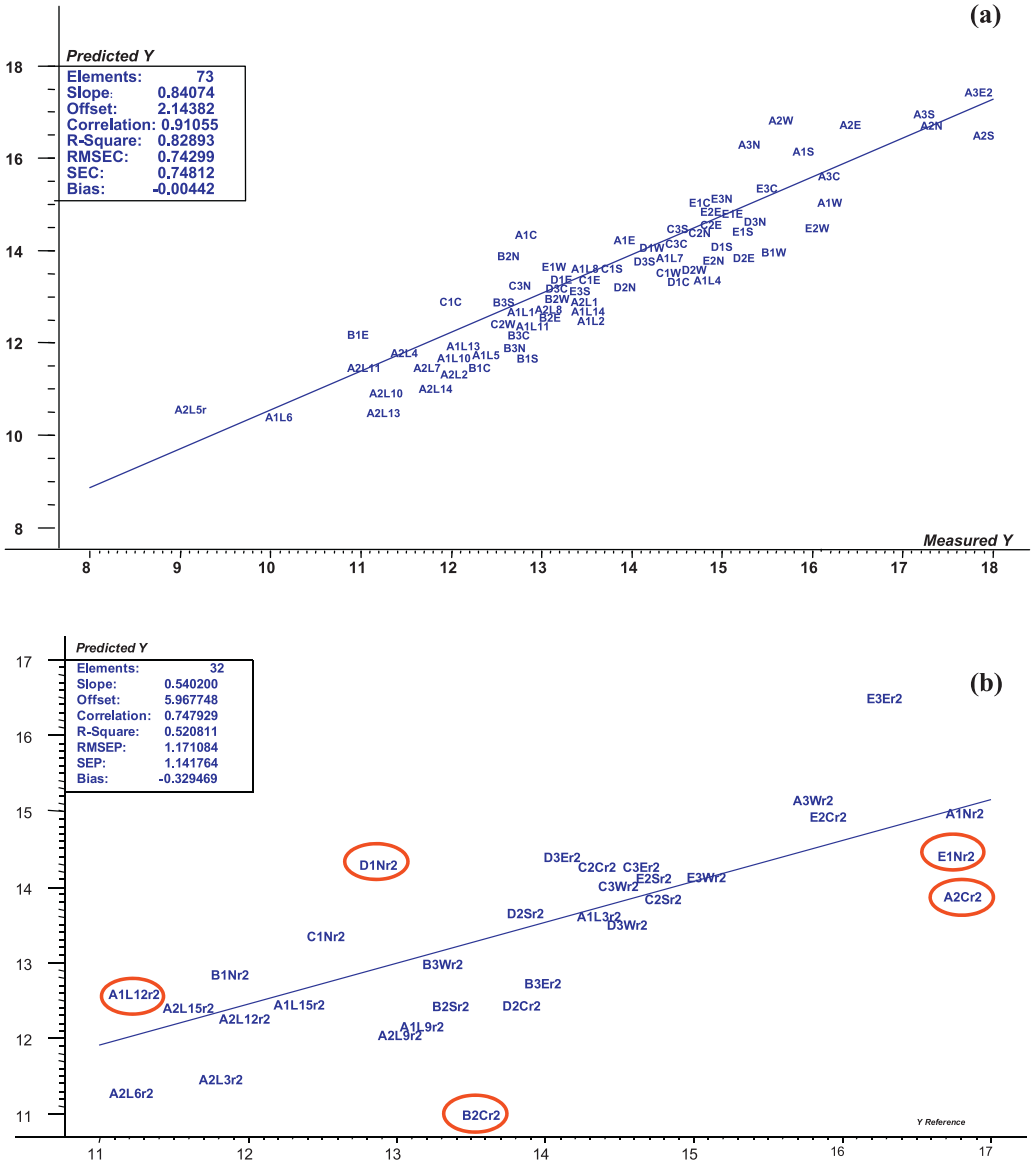

Fig. 4. Prediction of oleuropein content in olive leaves using MIR. Regression line of PLS models of oleuropein content: calibration set (a), prediction set (b). Circulated samples have an error >10%. RMSEC, root mean square error of calibration; SEC, standard error of calibration; RMSEP, root mean square error of prediction; SEP, standard error of prediction.

(phenols). The intense bands between 1150 and 950 cm^{-1} corresponds mainly to the endocyclic and exocyclic C–O stretching vibrations of carbohydrates.

3.3. Calibration and validation

A calibration model (Fig. 4) for predicting oleuropein content of olive leaves was built using the PLS method from MIR spectra. The calibration ($n = 73$) set was used for building model, while the validation set ($n = 32$) was used for testing the robustness of the developed model. The performance of the PLS model was evaluated in terms of standard error of calibration (SEC), root mean square error of calibration (RMSEC), standard error of prediction (SEP), root mean square error of prediction (RMSEP), correlation coefficient (R) and relative error of prediction (REP). Different spectral treatments were tested (SNV, MSC, second derivative, baseline correction) but no improved the results. Finally, the best model was obtained using first derived spectra and SNV correction on 1800–700 cm^{-1} spectral range.

Good correlation of calibration was found between MIR spectra and content of oleuropein with a coefficient of correlation around 0.91 (Fig. 4a). The prediction results were faintly good with a coefficient of correlation around 0.74 (Fig. 4b). Besides, the SEC (0.748%), the RMSEC (0.742%), the SEP (1.141%) and the RMSEP (1.171%) for oleuropein content are relatively low. The ability of MIR spectroscopy in prediction of oleuropein content was confirmed by the faintly good value of REP. In fact, REP, the relative error of prediction, represented about 8.5%.

The error values in absolute terms in the validation set ranged from 0.86% to 19.34%. An error of 10% in absolute term between the reference and the predicted values was considered as the limit of the significance of the difference. With this limit, five samples (A2C, E1N, B2C, D1N, A1L12) in the validation set were bad predicted for their oleuropein content. The samples coded A2C and E1N were bad predicted but their oleuropein contents were high (respectively 17.13 and 17.17%) which correspond to the high part of the calibration line. When the five samples (A2C, E1N, B2C, D1N, A1L12) were not in the prediction set, the SEP is equal to 0.73% with a R^2 to 0.88 which is satisfactory.

The developed PLS model can be considered satisfactory for rapid predictive oleuropein content in olive leaves.

4. Conclusion

Mid-infrared spectroscopy presents potential for a rapid quantification of oleuropein in olive leaves. MIR analysis could be an alternative to chromatographic analysis. This preliminary attempt is promising technique. The error of the assay is acceptable (<10%) for a rapid assessment of the oleuropein content of leaves.

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